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DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

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IIT Research Institute
Chicago, Illinois

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Annual Progress Report

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ABSTRACT

Studies were continued in efforts to elucidate the basic physiological mechanisms that may be involved in the attraction of mosquitoes to their warm-blooded hosts. Experimental observations were made to test the validity of the gamma-aminobutyric acid (GABA)-carbon dioxide (CO_2) hypothesis described in previous reports. This hypothesis could explain the neurochemical interactions that govern a mosquito's behavior in the presence of its host. It was postulated that the formation of carbamino-GABA underlies the activating effects of CO_2 on mosquitoes.

The isolated central nervous system of the cockroach, Periplaneta americana, was utilized as a model system to test the effects of carbamino-GABA and other compounds on the nervous system of an insect. GABA inhibited the spontaneous firing rate of this preparation in the absence of CO_2, but stimulated it in its presence. Gamma-hydroxybutyric acid, a substance which cannot form a carbamino compound, was inhibitory both in the absence and presence of CO_2. A chemical analogue of carbamino-GABA, N-acetyl GABA (NAG), stimulated the preparation. Evidence was obtained showing that NAG and GABA probably compete for the same site on the synaptic membrane. These results have important implications in terms of the mechanism by which CO_2 stimulates specialized insect nervous tissue. Further studies are in progress.

The evaluation of selected compounds for mosquito repellency by the electronic recording method was continued. Compounds were selected on the basis of our past approach, i.e. that volatile chemical analogues of GABA, in terms of electronic distribution, would affect the mosquito's responses to its host.

A statistically designed series of tests were performed to test the validity of some basic assumptions that are involved in using the electronic method for the assay of repellents. The results of these tests largely substantiated our confidence in this method.

FOREWORD

This is Report No. IITRI-L6021-20 (Annual Progress Report) on IITRI Project L6021, entitled "Development of an Orally Effective Insect Repellent." The report covers the period from November 1, 1968 through October 31, 1969.

This project is being sponsored by the U. S. Army Medical Research and Development Command, Office of The Surgeon General, Washington, D. C. 20315, under Contract No. DA-49-193-MD-2281 and is being conducted by IIT Research Institute, Technology Center, Chicago, Illinois 60616. Previous work under this contract was conducted by IIT Research Institute from May 1, 1962 through October 31, 1968.

The project leader for this program is Mr. Philip Kashin, under the administrative supervision of Dr. Mary Henry. The statistical analyses were performed by Mr. Merl L. Kardatzke, who also devised the computer program for determining the repellency index and the statistical confidence limits for the test compounds. Valuable suggestions and discussions for the physiological phases of the work were contributed by Dr. William F. Danforth, Biology Department, Illinois Institute of Technology. Dr. James C. Lambert of the Electronics Division of IIT Research Institute suggested the modified recording arrangement for the electrophysiological recordings described below. Mr. Anthony M. Gross provided technical assistance in the assay of repellents.

In conducting the research described in this report, the investigator adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences - National Research Council.

The citation of any trade names in this report does not constitute an official endorsement or approval of the use of such commercial hardware or software.

All repellency test data are recorded in IITRI Logbooks C18467 and C19669. The computer output sheets also form part of our permanent records.

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DEVELOPMENT OF AN ORALLY EFFECTIVE INSECT REPELLENT

I. INTRODUCTION

The objectives of this program are to develop better topical insect repellents than those currently available, and if possible, to develop an insect repellent that is effective when administered orally. Such repellents should afford more uniform and longer-lasting protection from bites of hematophagous insects. Success in this undertaking could result in a significant reduction of human suffering from disease and discomfort caused by insect bites.

In order to accomplish these goals, studies were continued during this year to elucidate the basic physiological mechanisms that may be involved in the attraction of hematophagous insects to their warm-blooded hosts. An understanding of this mechanism would permit the premeditated design of chemical compounds that could confuse and interfere with the responses of a hematophagous insect to its host.

The experimental rationale and approaches followed in the work during this report period were largely established in previous years (ref. 1 to 5). Gamma-aminobutyric acid (GABA), a substance known to cause inhibition in the transmission of nervous impulses across certain synaptic structures, was found in mosquitoes (ref. 2 to 5). It was hypothesized that the interactions of GABA with carbon dioxide, heat, and water vapor form the basis of mosquitoes' attraction to their hosts. Further evidence supporting the hypothesis that the formation of carbamino-GABA underlies the activating effects of CO_2 on mosquitoes was obtained from the effects of CO_2, GABA, and gamma-hydroxybutyric acid, and N-acetyl-GABA (a chemical analogue of GABA-CO_2) on the spontaneous firing rate of the isolated central nervous system of the American cockroach, *Periplaneta americana* (L).

The testing of volatile chemicals for repellency that are GABA analogues in terms of electronic configuration was continued. Compounds were selected on the basis of our past approach, i.e., that chemical molecules that have an electronic distribution similar to that of GABA or its analogues would effect the mosquito's responses to its host.

In evaluating candidate compounds for mosquito repellency, a method is utilized wherein the bite of a mosquito is electronically detected and recorded. The electronically recorded data, together with the percentage of mosquitoes that engorged blood during a test are compared with the same parameters derived from controls in a computerized statistical analysis to yield statistical confidence limits for the repellent efficacy of test compounds.

A statistically designed series of tests were performed to test the effects of age, time of day, and other parameters on the biting rate of mosquitoes on control and repellent-treated animals using the electronic method. This was done in order to test the assumption that the variables affecting the biting rate on untreated controls will also affect the repellent-treated cases in a parallel way.

This annual report details further progress in our investigations in these areas.

II. PHYSIOLOGICAL STUDIES

A. Modifications of Electrophysiological Methods

The previous method for recording the spontaneous electrical activity from the 3rd thoracic ganglia of the isolated central nerve cord of the American cockroach, *Periplaneta americana* (L.) (ref. 4) was modified to give better recordings from this preparation. We previously used a single-ended system, where one electrode was the ground. This was not the optimal arrangement for collecting the data, since the system was not balanced electrically. This resulted in an increased noise level and loss of sensitivity. Stray AC signals also proved to be a problem, even in the electrically shielded enclosure that is used for this work since some signals arose from the AC equipment within the enclosure. The stray AC signals were eliminated by partially shorting the recording electrode to the ground through a 10,000 ohm resistor. This procedure caused an even further reduction in sensitivity.

The circuitry is now changed so that the reference electrode is a true reference electrode with regard to the recording electrode, and the data-gathering electronic system is utilized in a true push-pull mode. A one megohm potentiometer was installed between the ground, the reference electrode, and the recording electrode as shown in Figure 1. The electrical balance between the reference and recording electrodes can be adjusted with the potentiometer in such a way that stray AC signals are virtually eliminated, while the signal strength is enhanced.

This is done as follows. The reference electrode is immersed into the saline solution in the 5 cc beaker, while the recording electrode with the ganglion chain (usually consisting of the 3rd thoracic ganglia and 3 or 4 abdominal ganglia) is hanging freely above the solution and not touching it. The potentiometer is turned until the AC signal is eliminated, as determined from the oscilloscope trace. At this point the electrodes are balanced and the electronic counter can be calibrated as described below.

It is important to standardize the method of setting the sensitivity level of the electronic digital counter which tallies the spontaneous action potentials from the nerve cord over the 5-sec counting interval. If the level is set too low, data will be missed; if set too high, too much noise will be recorded. The following method is used to set the sensitivity level of the counter. With the electrodes shorted in the saline solution, the counter sensitivity control is set to just barely show no counts when the preamplifier amplification control is set one step higher (more sensitive) than the setting that is used during the experiment. (Each amplification step on the Grass Model P-4 preamplifier that is used for this work is a multiple of 1.5). The preamplifier amplification control is then set to the level at which the experimental data will be taken (i.e. one step lower than the setting at which the zero level was calibrated). Using this procedure, a signal to noise ratio of approximately 1.5:1 is obtained, which is very satisfactory for this work.

Treatment of the nerve cord with chemicals has also been standardized. The test solution is placed in the 5 cc beaker, the beaker is raised by means of the jack so that the 3rd thoracic ganglia and recording electrode are completely emersed in the test solution for 5 sec. The beaker is then slowly lowered, until the surface tension just breaks from the recording electrode holding the ganglia. When the surface tensions breaks, the electrodes are unshorted, and the data gathering can proceed. The

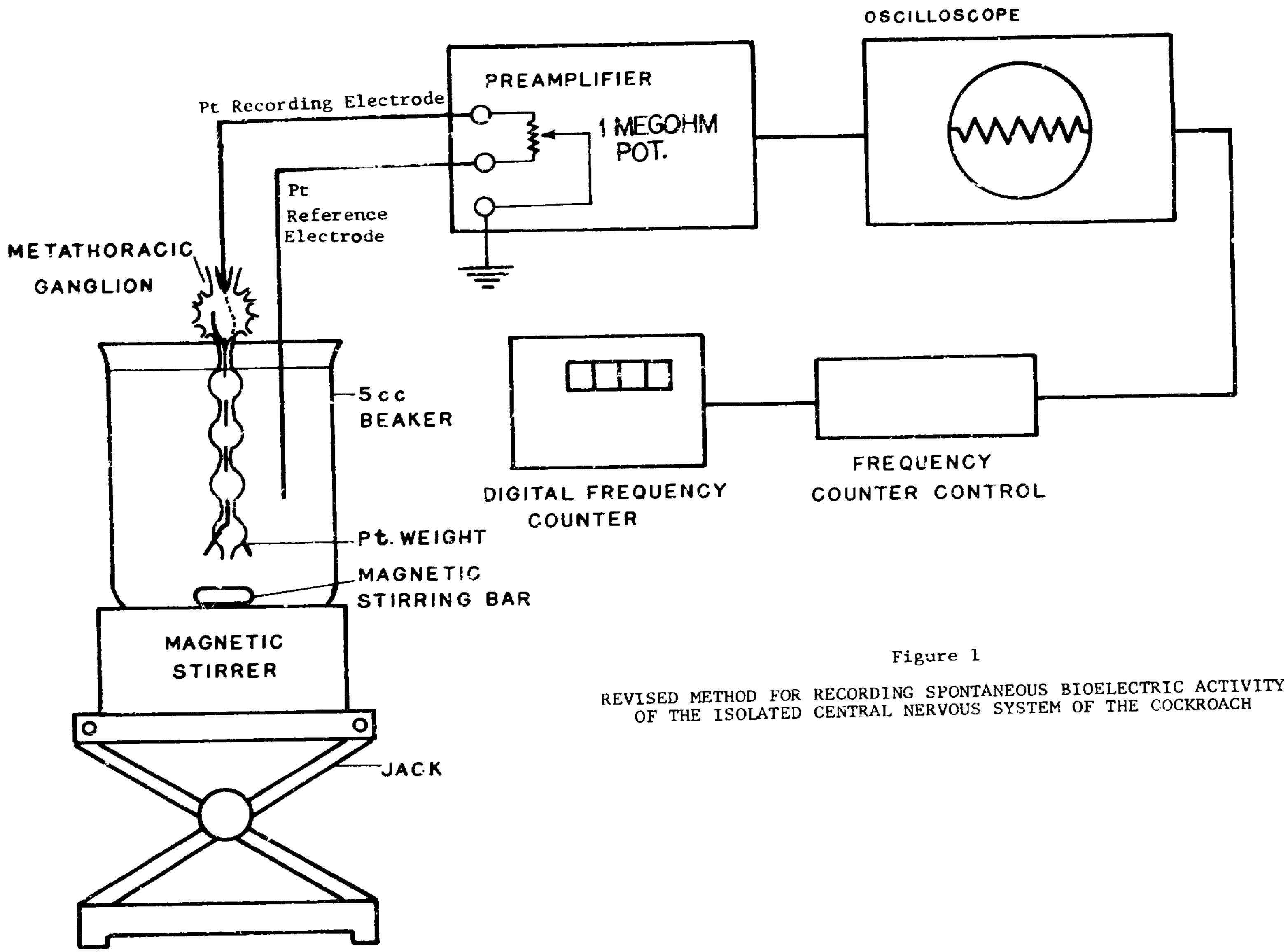


Figure 1

REVISED METHOD FOR RECORDING SPONTANEOUS BIOELECTRIC ACTIVITY OF THE ISOLATED CENTRAL NERVOUS SYSTEM OF THE COCKROACH moment of unshorting of the electrodes (when the surface tension breaks) can be observed visually, but is usually determined when the oscilloscope trace suddenly changes. This method has proven to be reproducible and accurate. Therefore, the amount of the nerve cord which is removed from the solution is carefully controlled. This is important, since the recorded discharge rate increases as more of the nerve cord is removed from the solution.

When the preparation is treated with a test solution, the 5 cc beaker containing the saline in which the preparation is suspended is lowered by means of the jack, and the saline beaker is replaced with the beaker containing the test solution. The test beaker is then raised to the preparation, and the entire preparation is immersed in the test solution for 5 sec. After this time, the 5 cc beaker is lowered until the surface tension breaks from the recording electrode as described above, and data is recorded. In the following figures, an arrow pointing to a double circle designates the last point of the data before the test solution was added. Therefore, immediately after this point there is actually a discontinuity during which the above-mentioned operations were performed.

B. Modified Physiological Saline Solutions

Another modification in the experimental procedure was made. Bicarbonate buffer was omitted from the saline solution and a new buffer substituted. This buffer, N-2-hydroyethylpiperazine-N'-2-ethane-sulfonic acid (HEPES), has a pK value of 7.55, and has been successfully used in tissue culture and biochemical work. This buffer was used since bicarbonate buffer can give rise to CO_2. The new buffer contributes no bicarbonate to the system, and has a pK value which is in the physiologic range.

The buffered saline solution we now use has the following composition. All quantities are in terms of grams/liter.

NaCl	9.1
KCl	0.9
$CaCl_2$	5.07
$MgCl_2.6H_2O$	0.8
Glucose	4.0
HEPES buffer	2.383 (0.01 M)

The completed solution is carefully titrated to pH 7.60. A concentrated stock solution (10x) is diluted and titrated as needed. The above saline is a modification of that described by Smit et al (ref. 6). This buffered saline solution keeps the preparation in good condition for many hours. The following experiments were carried out with all the modifications described above.

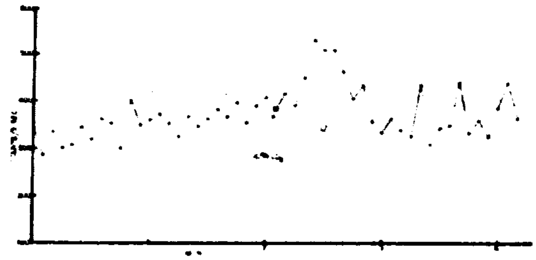
C. Experimental Results and Discussion

1. Studies of the Effects of Carbon Dioxide, GABA, and Carbamino-GABA on the Insect Central Nervous System

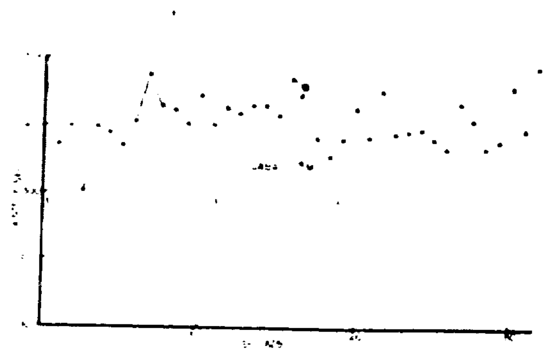
Figure 2a shows the stimulatory effects of the saline solution alone treated with CO_2. Figure 2b shows that 10^{-6} M GABA has practically no neuroinhibitory action, but when treated with 10% CO_2 (Figure 2c) appears to be somewhat more stimulatory than the saline-treated CO_2 solution (Figure 2a). At a GABA concentration of 10^{-4} M there may be a slight indication of possible inhibition (Figure 2d) but in the presence of 10% CO_2 (Figure 2e) a high degree of stimulation is shown that seems to be greater than that seen in Figure 2c.

Treatment of the preparation with 5×10^{-4} M GABA (Figure 3a) again possibly causes a slight immediate inhibition, but the preparation is greatly stimulated when the solution is treated with 10% CO_2.

Application of 10^{-3} M GABA causes an immediate and precipitous decline in the electrical activity of the preparation (Figure 3c) but this GABA solution becomes a potent stimulator when treated with 10% CO_2 (Figure 3d). Continuing these observations, it can be seen that treatment of the ganglia with 5×10^{-3} M GABA again causes an immediate decline in the firing rate (Figure 3e) but when the solution is treated with 10% CO_2 the control level of firing is maintained for almost a full minute before inhibition sets in (Figure 3f). Figure 4a shows the effects of treating at 10^{-2} M GABA with 10% CO_2. Although there was an immediate decline in discharge rate when treated with 10^{-2} M GABA (not shown), there is no decline for at least 30 sec after application of the CO_2-treated solution. All of the above experiments were performed on a single preparation.



(a)



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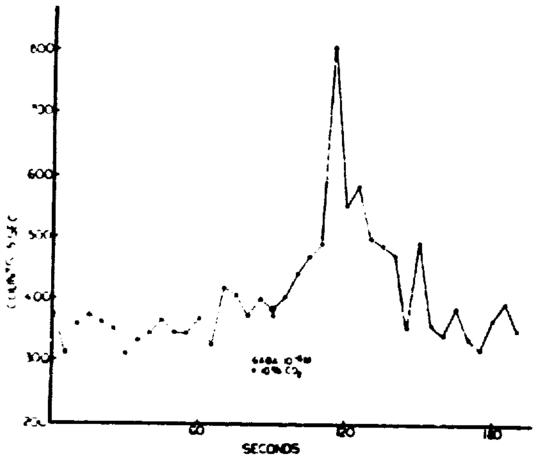
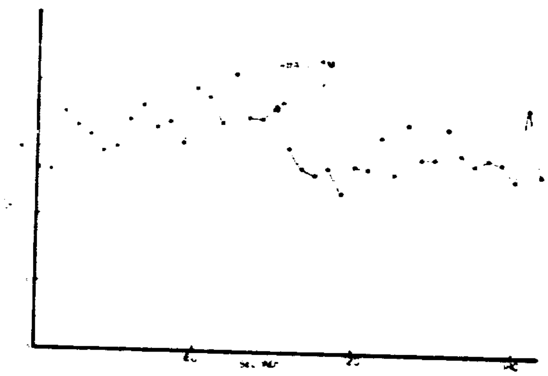
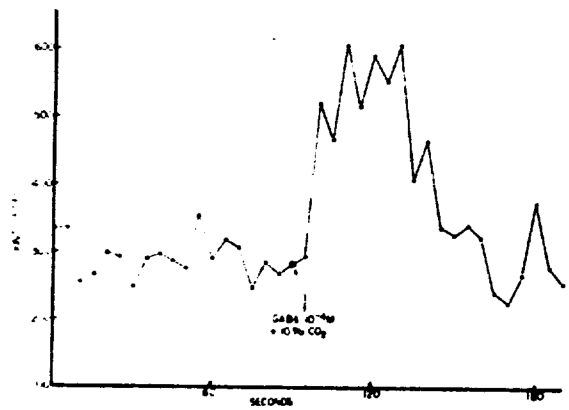


(c)



(d)



(e)

Figure 2

EFFECTS OF (a) CO_2, (b) GABA 10^{-6} M, (c) GABA 10^{-6} M + 10% CO_2, (d) GABA 10^{-4} M, AND (e) GABA 10^{-4} M + 10% CO_2, ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED COCKROACH CNS

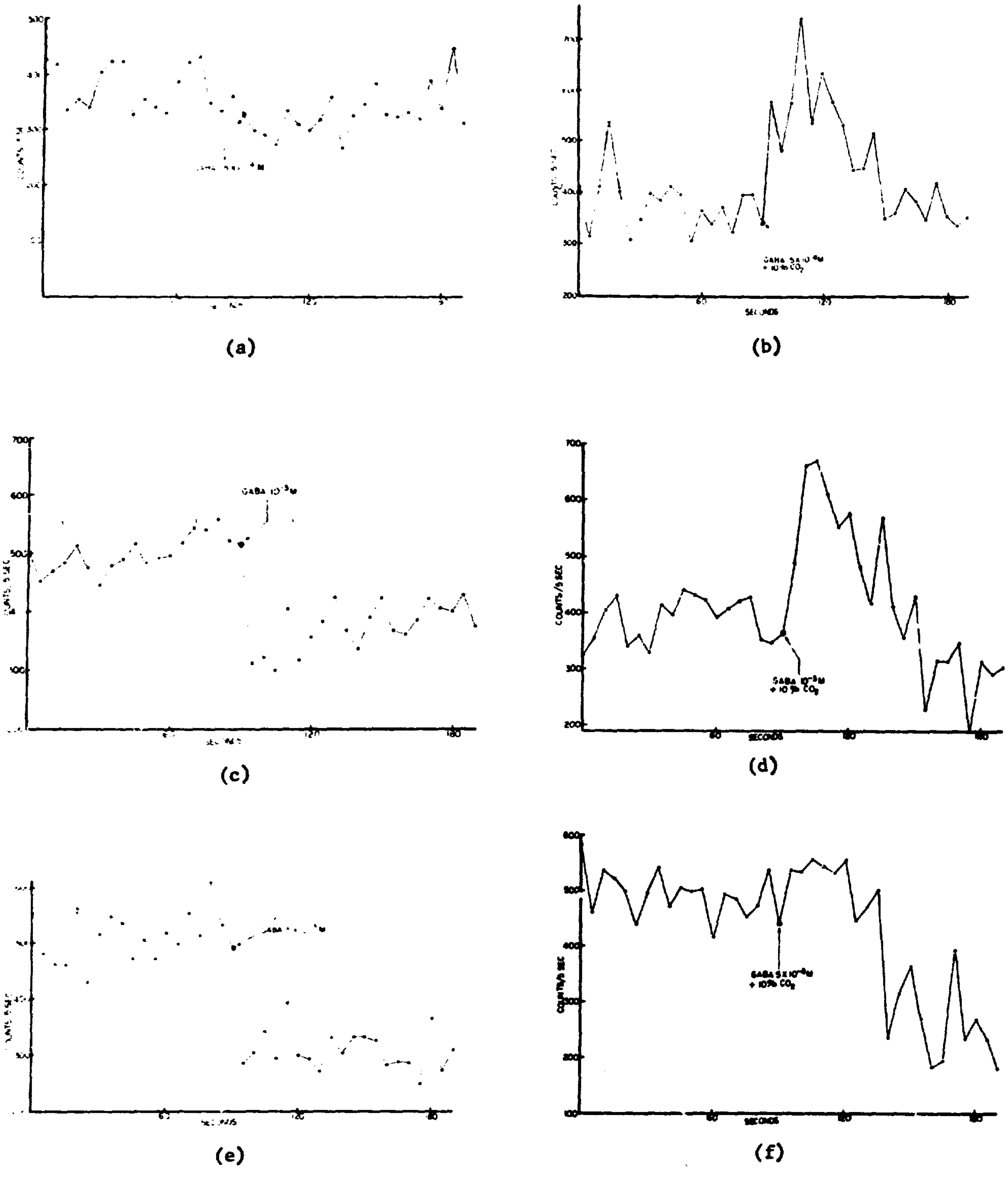
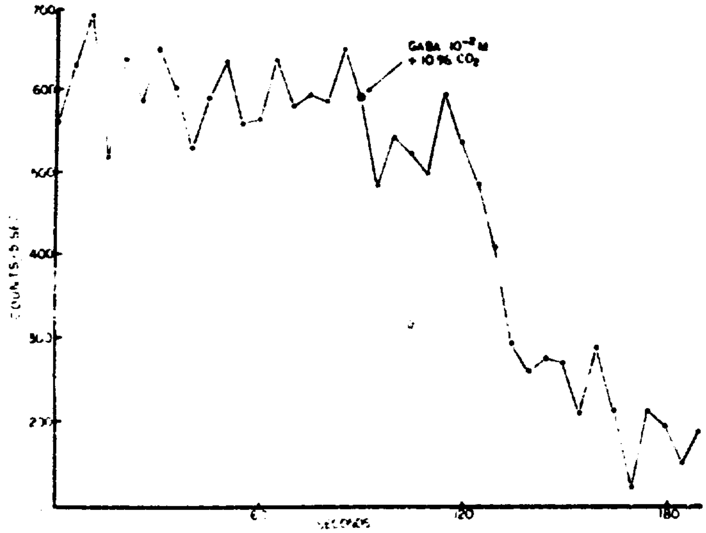
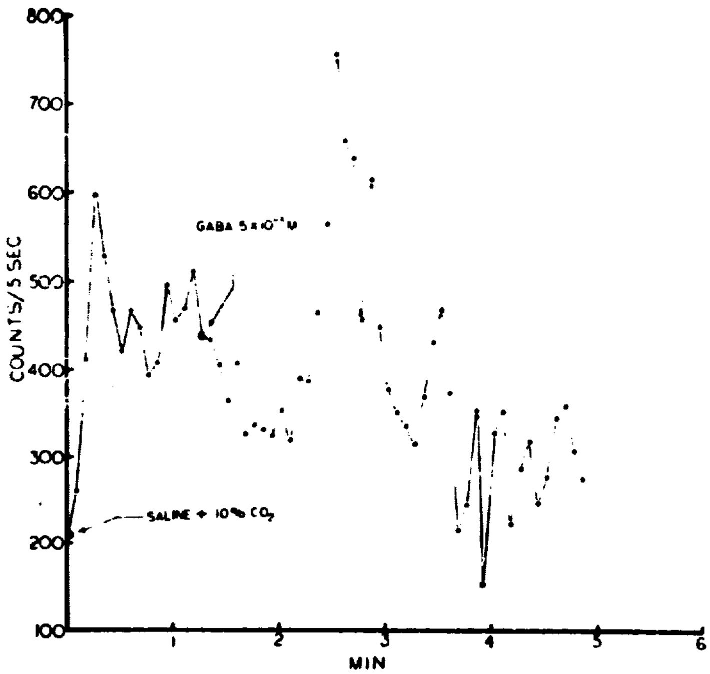


Figure 3

EFFECTS OF (a) GABA 5 x 10^{-4} M, (b) GABA 5 x 10^{-4} M + 10% CO_2, (c) GABA 10^{-3} M, (d) GABA 10^{-3} M + 10% CO_2, (e) GABA 5 x 10^{-3} M, AND (f) GABA 5 x 10^{-3} M + 10% CO_2, ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED COCKROACH CNS



(a)



(b)

Figure 4

EFFECTS OF (a) GABA 10^{-2} M + 10% CO_2 AND (b) SALINE + 10% CO_2 (BEFORE AND AFTER THE ADDITION OF GABA 5×10^{-3} M), ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED COCKROACH CNS

It was also of interest to observe what the effects would be on a preparation that was first treated with saline that had CO_2 bubbled into it, and into which an untreated GABA solution was introduced to give a final concentration of 5×10^{-3} M. It can be seen in Figure 4b that the preparation is stimulated after about 30 sec to a greater level than that of CO_2 alone, followed by a gradual decline.

The amount of decline after the initial stimulation in the CO_2-treated GABA solutions, having concentration of 5×10^{-4} M GABA or less, is to the control level, while the decline goes below control levels in the more concentrated CO_2-treated GABA solutions. This corresponds with the observations that inhibition at GABA concentration of 5×10^{-4} or below is hardly discernible, while inhibition at GABA levels of 10^{-3} M and higher is clearly evident. Though a definite stimulation is seen immediately upon lifting the ganglia from the solution, it appears that with time the full inhibitory action of GABA is established. This is probably due to the rapid escape of CO_2 from the preparation after removal from the solution to record the data. As the GABA concentration increases, the inhibitory effect of GABA is established at the higher concentrations with the escape of CO_2.

It should be mentioned that even if it were feasible to perform these experiments without removing the ganglia from solution and exposing it to air (i.e. if the problem of the short-circuiting of the electrodes in solution could be overcome), it would still not be possible to do the experiments in this way. This is because if the ganglia is continuously immersed in solution, it soon becomes anoxic and the spontaneous discharges cease altogether.

2. Studies of the Effect of Carbon Dioxide and GHB on the Insect Central Nervous System

A parallel series of experiments were performed with gamma-hydroxy butyric acid (GHB). This compound was of interest because it is a neuroinhibitor (ref. 7,8) although it is less inhibitory than GABA (ref. 7,9). GHB has a chemical structure similar to that of GABA, with the exception that it possesses a gamma-hydroxy group instead of a gamma-amino group. Since GHB does not possess an amino group, it cannot form a carbamino compound with CO_2 and therefore can be used to test the hypothesis that carbamino-GABA formation is the mechanism by which CO_2 stimulates the nervous system of the insect. If the mechanism of stimulation by CO_2 is not that of carbamino formation, but instead through an independent receptor site for CO_2, then the results of the GHB experiment would be similar to those of the GABA experiment.

Representative results of this experiment are shown in Figures 5 (a-f) and 6 (a-e). It can be seen that the stimulatory affects of CO_2 steadily decline as the concentration of GHB increases.

No inhibition is evident at GHB concentrations of 10^{-6} M (Figure 5a), 10^{-4} M (Figure 5c), 10^{-3} M (Figure 5e) or 5×10^{-3} M (Figure 6a). The stimulatory effects of CO_2 are at a maximum at GHB 10^{-6} M + 10% CO_2 (Figure 5b), decreases at a GHB concentration of 10^{-4} M + 10% CO_2 (Figure 5d), and are completely abolished at GHB concentrations of 10^{-3} M + 10% CO_2 (Figure 5f) and 5×10^{-3} M GHB + 10% CO_2 (Figure 6b).

It is seen in Figure 6c that not until a GHB concentration of 5×10^{-2} M is reached can the inhibitory effects of GHB be discerned, and that the addition of 10% CO_2 to this solution (Figure 6d) has little or no modifying effect on the inhibition of this concentration of GHB. After this series of experiments the preparation was washed with saline, and then treated with a saline solution containing 10% CO_2 (Figure 6e). It can be seen that the preparation has completely lost its sensitivity to CO_2 after the GHB treatment. Although not shown in this series of experiments, we previously reported (ref. 9) that the loss of sensitivity of the preparation to CO_2 could be completely reversed by subsequently treating the preparation with GABA.

These findings lend strong support to the suggestion that carbamino-GABA formation is the mechanism by which CO_2 stimulates insect nervous tissue which is sensitive to the action of CO_2, and to the hypothesis that carbamino-GABA is a neurostimulator in certain specialized insect neuro-receptors.

It is also of interest to note that although GHB is about 1 to 2 orders of magnitude less inhibitory than GABA, and does not demonstrate any significant neuroinhibitory action at concentrations less than about 5×10^{-2} M, the stimulatory effects of CO_2 show considerable decline in the presence of 10^{-4} M GHB (Figure 5d) and are totally abolished at a GHB concentration of 10^{-3} M (Figure 5f). It thus appears that GHB even at relatively low concentrations can successfully compete with endogenous GABA for the synaptic receptor site, and prevent the GABA-CO_2 formed from endogenous GABA (in the case of GHB solutions treated with CO_2) from reaching the receptor site. This brings up the question of relative affinities for the synaptic receptor site, and related to this, whether there is one receptor site or more than one such site for CO_2, GABA, GABA-CO_2, and GHB. The following experiments represent our first attempts to answer these questions.

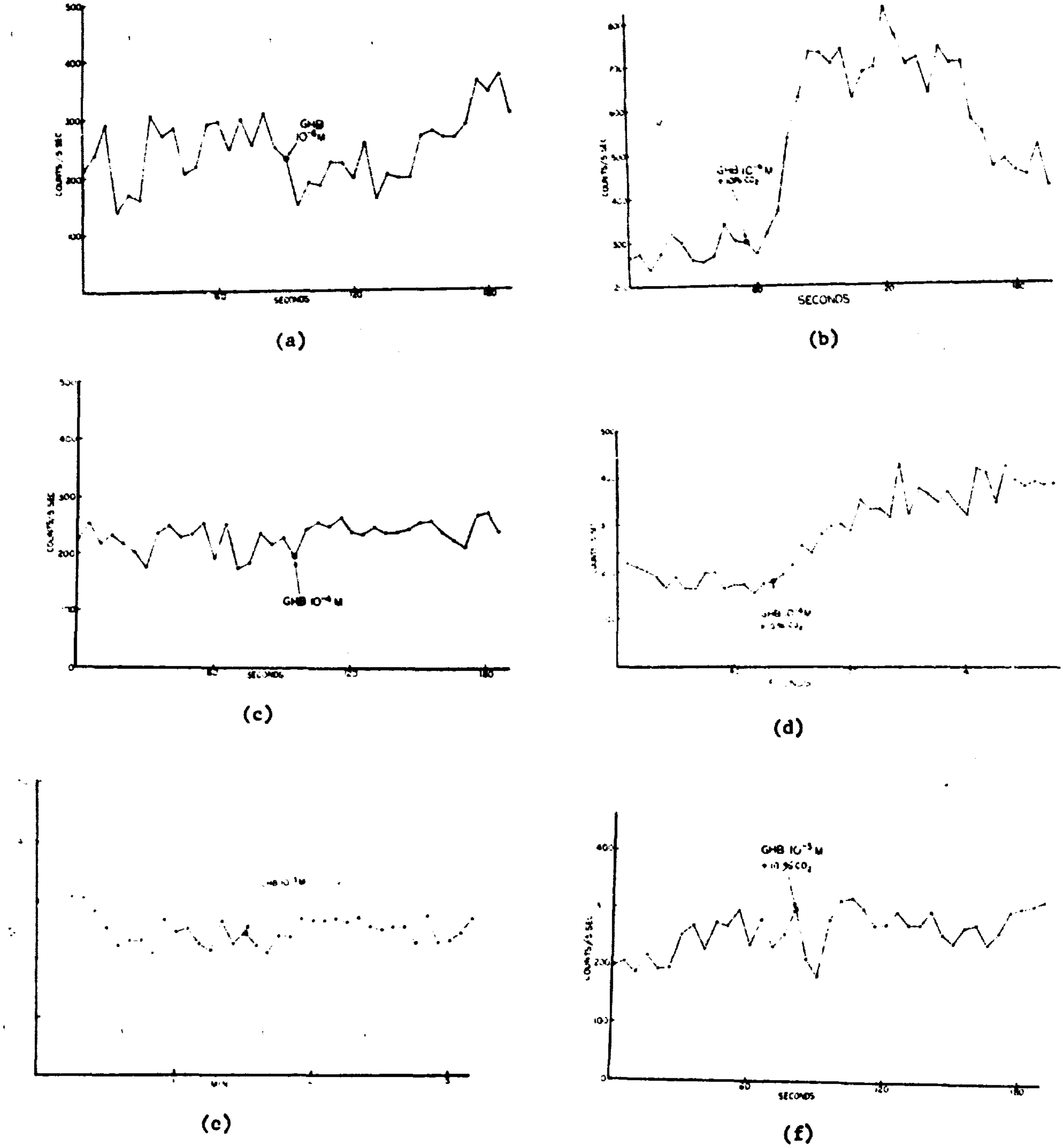


Figure 5

EFFECTS OF (a) GHB 10^{-6} M, (b) GHB 10^{-6} M + 10% CO_2, (c) GHB 10^{-4} M, (d) GHB 10^{-4} M + 10% CO_2, (e) GHB 10^{-3} M, AND (f) GHB 10^{-3} M + 10% CO_2, ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED COCKROACH CNS

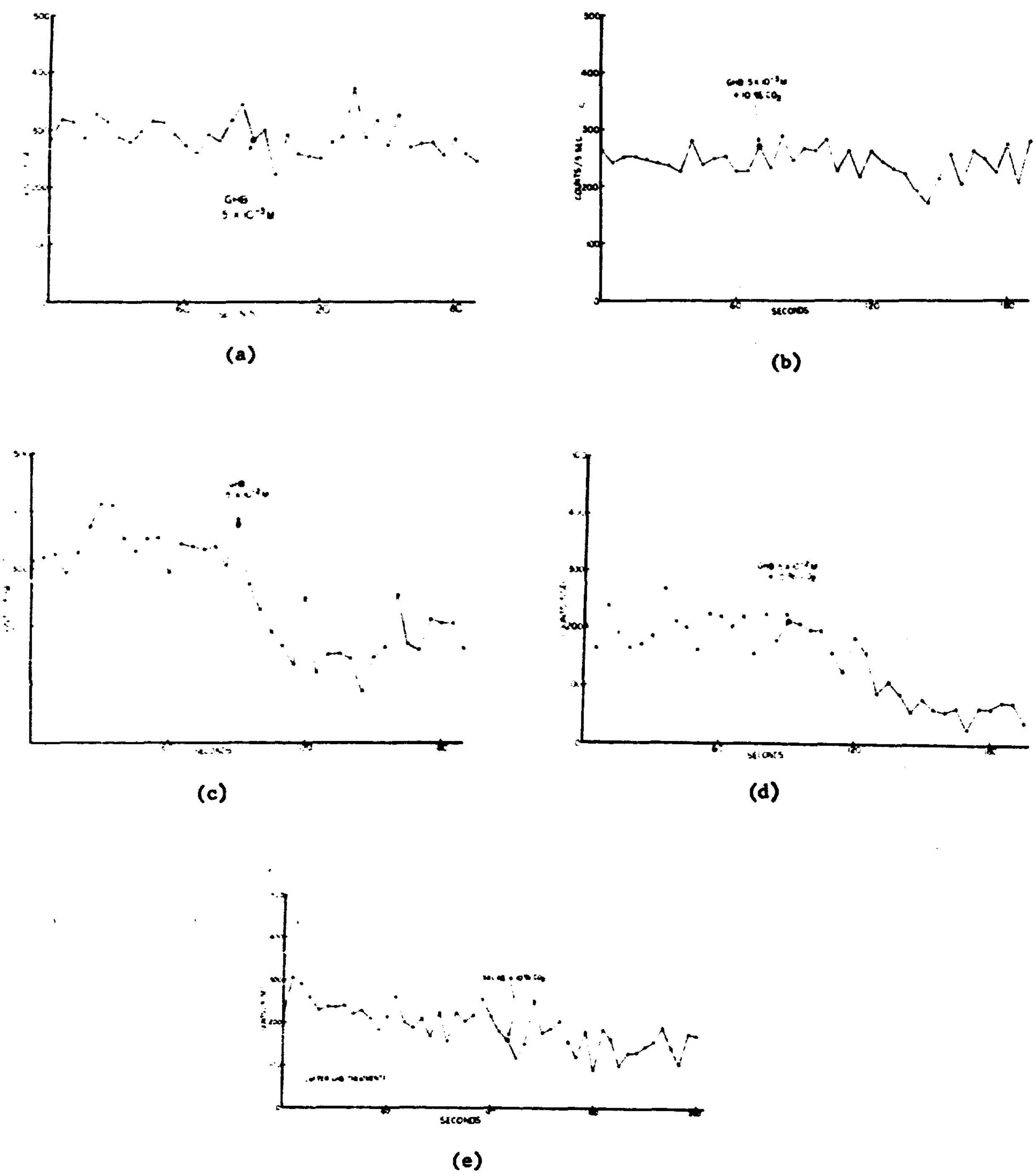


Figure 6

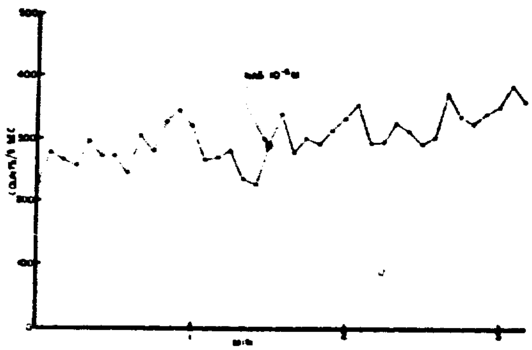
EFFECTS OF (a) GHB 5×10^{-3} M, (b) GHB 5×10^{-3} M + 10% CO_2, (c) GHB 5×10^{-2} M, (d) GHB 5×10^{-2} M + 10% CO_2, AND (e) SALINE + 10% CO_2 AFTER GHB TREATMENT, ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED COCKROACH CNS

3. Studies on the Site of Action of CO_2, GABA, and Related Compounds

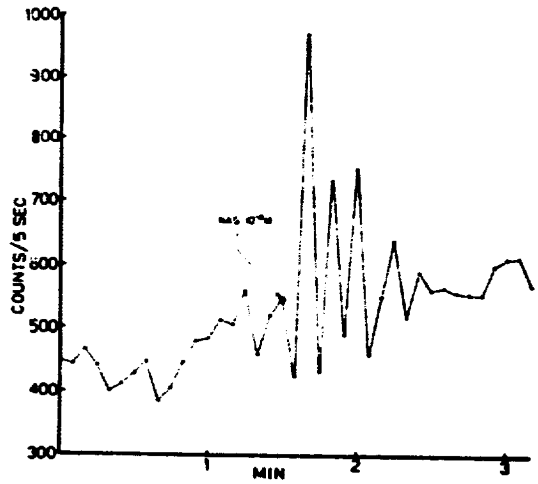
The possibility of using N-acetyl-GABA (NAG) as a chemically stable analogue of GABA-CO_2 was previously discussed, and this compound was shown to be stimulatory (ref. 4). A series of representative experiments (Figure 7a-c) show how the electrical discharge rate of the ganglion is affected as the concentration of NAG is increased. There is little or no stimulation at an NAG concentration of 10^{-6} M (Figure 7a). At a concentration of 10^{-4} M NAG (Figure 7b) a definite increase in the discharge rate is observed, and a prominent oscillatory pattern appears. At a concentration of 10^{-1} M NAG (Figure 7c), the preparation discharges at a very high and relatively sustained rate, and again shows a pronounced oscillatory discharge pattern over and above the increase in the overall discharge rate. The decline in discharge rate after about one minute may be due to exhaustion of the preparation.

If NAG is indeed a valid analogue of GABA-CO_2, then it is possible to test whether GABA and GABA-CO_2 compete for the same site on the synaptic membrane of the cockroach CNS. Since GABA-CO_2 is a very unstable compound under these experimental conditions, NAG could be substituted for GABA-CO_2 to observe the effects on the discharge rate of the preparation by varying the concentration of NAG in the presence of a fixed amount of GABA.

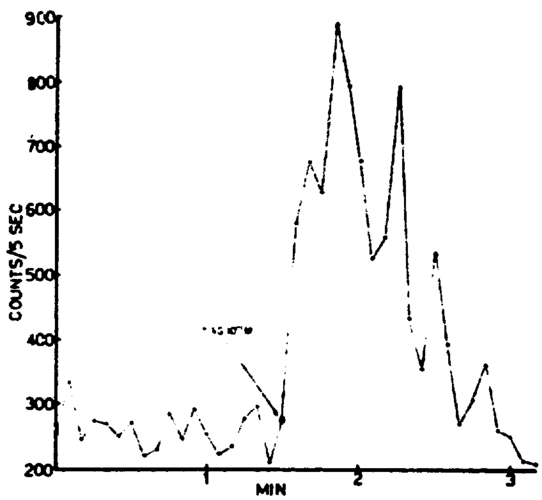
The results of partially completed experiments designed to test these effects are shown in Figure 8 (a-d) and Figure 9 (a-c). Figure 8 shows the results of treating one preparation with a constant concentration of GABA (10^{-4} M) and varying concentrations of NAG. At a GABA concentration of 10^{-4} M and an NAG concentration of 10^{-4} M, neither inhibition due to GABA nor excitation due to NAG is seen. The lack of GABA inhibition at 10^{-4} M corresponds with what was observed by treatment with this same concentration of GABA in a previous experiment (Figure 2d). However, excitation was previously seen with this concentration of NAG (Figure 7b). Thus, the stimulatory effects of NAG are definitely modified in the presence of a concentration of GABA that is barely sufficient to effectively inhibit the bioelectric activity of the preparation. These results could be explained if these two substances compete for the same site.



(a)



(b)



(c)

Figure 7

EFFECTS OF VARIOUS CONCENTRATIONS OF NAG ON THE SPONTANEOUS DISCHARGE RATE OF THE ISOLATED COCKROACH CNS

(a) NAG 10^{-6} M; (b) NAG 10^{-4} M; (c) NAG 10^{-1} M

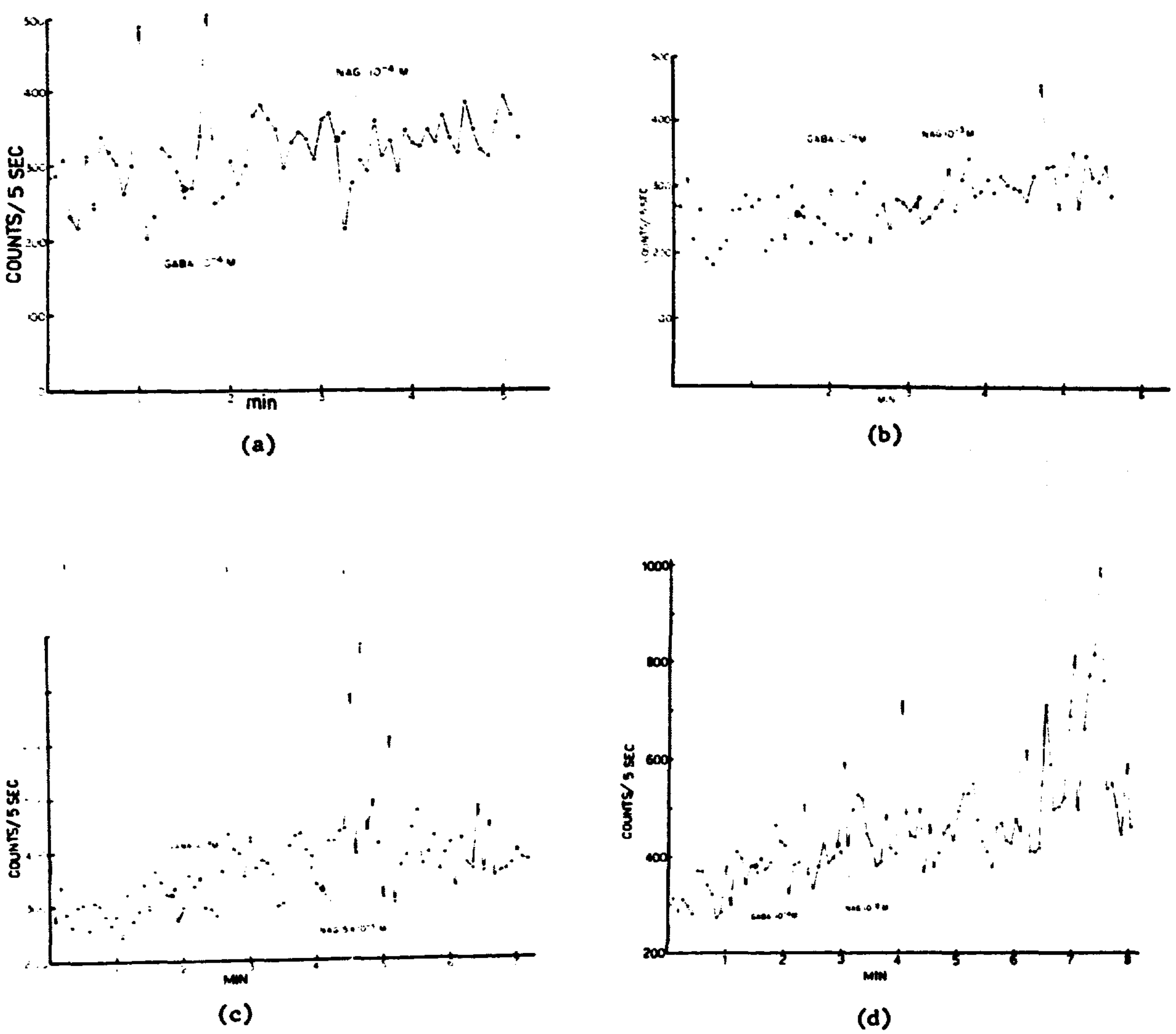
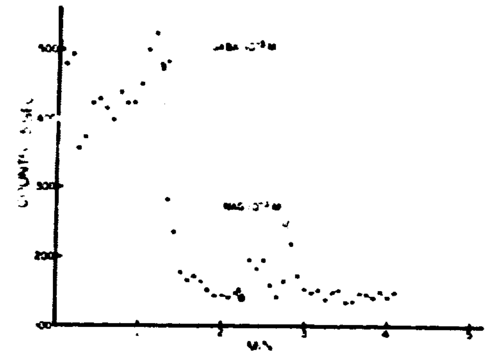
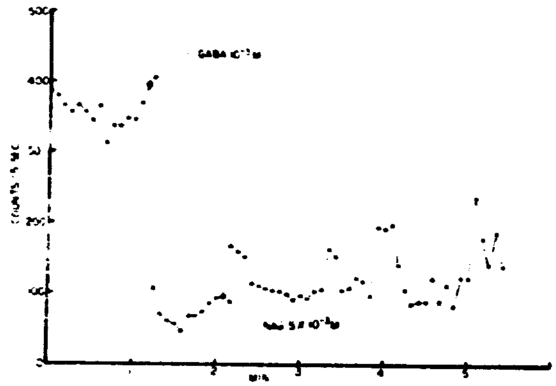


Figure 8

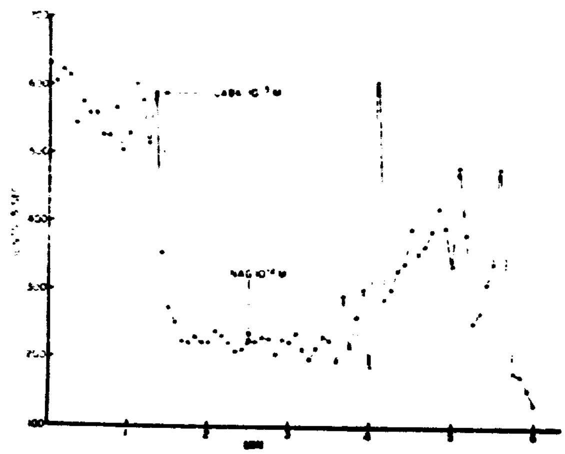
EFFECTS OF ADDING (a) NAG 10^{-4} M, (b) NAG 10^{-3} M, (c) NAG 5×10^{-3} M, AND (d) NAG 10^{-2} M, TO THE COCKROACH PREPARATION DURING TREATMENT WITH 10^{-4} M GABA



(a)



(b)



(c)

Figure 9

EFFECTS OF ADDING (a) NAG 10^{-3} M, (b) NAG 5×10^{-3} M, AND (c) NAG 10^{-2} M, TO THE COCKROACH PREPARATION DURING TREATMENT WITH 10^{-3} M GABA

Figure 8b shows the effects of 10^{-3} M NAG in the presence of 10^{-4} M GABA. With this mixture there does appear to be some stimulation when NAG is added. In the mixture that contains 10^{-4} M GABA and NAG at 5×10^{-3} M, a stimulation with an oscillatory discharge pattern becomes clearly evident (Figure 8c) and as the NAG concentration is further increased to 10^{-2} M in the presence of 10^{-4} M GABA (Figure 8d), the stimulation and oscillatory discharge patterns are further increased and sustained.

In order to further test these effects, the experiment was repeated using a constant GABA concentration of 10^{-3} M. This amount of GABA was previously shown to have a definite inhibitory effect on the discharge rate of the preparation (Figure 3c). It can be seen in Figure 9 (a-c) that treatment of another preparation with 10^{-3} M GABA definitely decreases the discharge rate of the preparation in all cases. Addition of NAG at a concentration of 10^{-3} M barely affects this inhibition (Figure 9a). A greater stimulation is seen when a concentration of 5×10^{-3} M NAG is added to the GABA solution (Figure 9b), and the preparation is highly stimulated after a lag of about 1.5 min with the addition of 10^{-2} M NAG to the GABA (Figure 9c), and an oscillatory discharge pattern is again discernible.

Though other experiments remain to be done in this series, some interesting observations can be made. It appears that NAG and GABA do indeed compete for the same site, since increasing the GABA concentration delays the time of onset for the expression of the stimulatory effects of NAG. The concentration dependence of these effects, together with the fact that a noninhibitory concentration of GABA seems to decrease the sensitivity of the ganglion to NAG, indicates that these two substances are competing for the same site. If NAG is a valid analogue of GABA-CO_2, then it follows that GABA-CO_2 also acts on the same receptor site. If this is indeed the case, then a molecule of GABA, a neuroinhibitor on the receptor site, can in the presence of CO_2 immediately become a neurotransmitter by forming the carbamino compound at the same receptor site. The only rate-limiting step controlling this respo se is the diffusion rate of CO_2 in the tissue, and not that of the larger and more polar molecule of GABA-CO_2. Therefore the response to increased CO_2 tension in specialized insect nervous tissue is directly controlled by the partial pressure of CO_2, and almost immediately expressed.

From data on the rate constants of the reaction of beta-alanine with CO_2 (ref. 10) it can be calculated that the half-life of the carbamino compound is about 140 milliseconds. Beta-alanine is structurally very similar to GABA, differing from GABA by only one carbon atom between the carboxyl and amino groups. If the half-life of the carbamino-GABA compound is of the same order of magnitude as that of carbamino-beta-alanine, then it seems clear that the carbamino-GABA compound will be extremely responsive to CO_2-tension, and will exist only as long as the partial pressure of CO_2 is sufficiently high for its formation in effective quantities. It will spontaneously and rapidly decompose when the partial pressure of CO_2 decreases.

Thus, an automatic and highly sensitive neurochemical mechanism is available to the insect for the control of CO_2 tension. Increased nervous activity in specialized receptor structures due to increased CO_2 concentration and carbamino-GABA formation could ultimately lead to the initiation of appropriate responses to control CO_2 tension. Such receptors could, for instance, be advantageously situated in the neural apparatus for the control of insect ventilation. In this regard, it is of interest to note again the oscillatory patterns previously mentioned that are exhibited by the preparation when treated with NAG and the NAG-GABA-mixtures. It is possible that these oscillations are actually reflections of an intrinsic "breathing" or ventilatory neural discharge pattern that is triggered by the various treatments.

It has long been known that respiratory movements in the insect are under both nervous and chemical control (ref. 11). Respiratory bursts in the isolated nerve cord of the praying mantis occur with the same frequency as the normal resting ventilatory movements of the intact insect (ref. 12).

The actions of the spiracles are coordinated by the central nervous system in P. americana. CO_2 affects the respiratory system in this insect and other cockroaches by a direct action on the ventral nerve cord, which results in ventilatory movements (ref. 13). Guthrie and Tyndall (ref. 13) state, "The presence of sensory structures in the spiracles, responsive to relatively low tensions of CO_2, suggests a useful local control mechanism, in addition to the central one in the nervous system, which could be used to select appropriate ventilation flow."

If it is established in principle that carbamino-GABA is a stimulatory neurotransmitter as we have postulated, then the formation of this substance as a direct function of CO_2 concentration has obvious relevence to the fundamental mechanisms governing the nervous control of ventilation in insects. The results of these investigations also support the hypothesis that originally motivated this work, i.e., that the formation of GABA-CO_2 in specialized neural receptors of the mosquito is the mechanism whereby increased CO_2 tension stimulates mosquito activity and triggers host-seeking behavior (ref. 5).

It will be our immediate goal in future investigations to test the effects on the preparation of mixtures of NAG with higher and lower GABA concentrations in order to further observe these trends. We will also observe the effects of mixtures of GHB with NAG which could yield further information regarding the site of action of these substances and relative affinities for the site. Furthermore, since Kerkut et al (ref. 14) postulated that glutamate may actually be the stimulatory transmitter in cockroach myoneural junctions, we will test the effects of glutamate, and mixtures of glutamate and GABA. Glutamate closely resembles GABA-CO_2 structurally (ref. 4), and it will therefore be of interest to determine whether glutamate and GABA-CO_2 act at the same site.

III. STATISTICAL TESTS OF "BITOMETER" METHOD

Our basic assumptions in applying the "bitometer" method to test mosquito repellents are:

a. Factors that affect the biting rate of mosquitoes on untreated control mice will also effect the biting rate of mosquitoes on repellent treated mice.

b. There are no differences between the data output of the various meters used in these tests.

c. The utilization of the electronic biting data, i.e., the amount of actual biting time as electronically recorded (P), makes an independent and significant contribution to total assay of repellency, regardless of percent mosquito engorgement (E).

Some support for the validity of the last assumption was obtained when it was revealed in a discriminant function analyses (ref. 2) that the parameter for the percentage of actual biting time (P) makes a significant contribution to the total assay of repellency. Therefore, this parameter was used to determine the repellency index by adding P to that of the percentage of engorged mosquitoes (E). However, we have never tested P independently to analyze interaction. The other two assumptions were never tested.

In order to further document our repellency testing method, and in view of the observations by Gouck and Smith (ref. 15) that mosquito age and time of day influences the biting rate of low-level repellent treated subjects, we designed a comprehensive statistical plan to test this effect, as well as some other possible effects in our repellency assay method. This experimental design allowed for independent analysis of the following.

1. A reevaluation of our previous findings of day-to-day variations in control tests (day effect).

2. A comparison with the result of Gouck and Smith (ref. 15) showing the effect of mosquito age and time of day on avidity in the presence of low-level repellent treatment.

3. The consistency of the responses among the three "bitometer-timers" we use in our testing procedures.

4. The influence of the actual number of mosquitoes in the test upon the test results.

5. The possibility of variations among different batches of mosquitoes.

The test was arranged with 14 days of testing. Each test day consisted of 4 sets, 2 in the morning, and 2 in the afternoon. Each set utilized 2 repellent treated mice and 1 untreated control. Each set also contained 2 different ages of mosquitoes run in parallel at different times of day on the 3 available meters.

Sixteen separate batches of mosquitoes were utilized to provide mosquitoes of ages 2, 4, 6, 8, 10, 12, 14, and 16 days of age in a randomized block design. The complete design calls for 56 untreated controls and 112 repellent treated tests, and the results were evaluated for the test and control observations. This experiment was designed to supply a comprehensive basis for future evaluations or applications to mass screening of repellents.

The design of the test is shown in Table 1. It is a randomized block design, and each mosquito age is tested at least once with each meter and at different times of day. Breeding of mosquitoes was carefully controlled so that emergence occurred at the proper time to match the age schedule. Lighting was automatically controlled to give 11 hours light and 11 hours darkness, with one hour for slow dimming and one hour for slow brightening of the lights to simulate dusk and dawn.

Dimethyl phthalate was used as the test repellent. One square inch of the belly of the test mice was treated with 0.5 cc of acetone containing 0.05 mg of repellent, while control mice were similarly treated with acetone alone. Only the treated areas of the mice were exposed to the mosquitoes. The test methods were as previously described (ref. 16,17).

The summary table (Table 2) shows the mean and 95% confidence limits for the control and the test observations for the variable age and time. Mosquitoes aged six days or less showed a lower level of response in the controls than more mature adults. The response in the treated group was consistent with this pattern, but did not show enough variation to be significant. Figure 10 shows this age effect graphically.

Table 2 also shows the repellency index, the sum of P plus E, for tests done at different times of day. The controls showed repellency index increasing throughout the day. In the repellent treated test group, the response is very low with the first morning test. It is lower than the other three (the second morning test and both afternoon tests). The variation in the other three tests is not statistically significant. Figure 11 graphs the effects of time of day for control and repellent treated cases.

Table 1

RANDOMIZED BLOCK DESIGN FOR TESTING EFFECTS OF MOSQUITO AGE, TIME OF DAY, AND OTHER PARAMETERS ON THE MOSQUITO BITING RATE ON REPELLENT-TREATED AND CONTROL CASES

Test Day	AM[a] Test 1 Meter 1	AM Test 1 Meter 2	AM Test 1 Meter 3	AM Test 2 Meter 1	AM Test 2 Meter 2	AM Test 2 Meter 3	PM[b] Test 1 Meter 1	PM Test 1 Meter 2	PM Test 1 Meter 3	PM Test 2 Meter 1	PM Test 2 Meter 2	PM Test 2 Meter 3
	Mosquito Age (days)			Mosquito Age (days)			Mosquito Age (days)			Mosquito Age (days)		
1	C[c]	12	10	6	C	4	10	C	12	C	6	4
2	C	14	12	C	2	8	14	C	12	8	C	2
3	10	16	C	C	14	2	2	14	C	C	10	16
4	C	4	2	8	12	C	C	12	8	2	C	4
5	4	10	C	6	16	C	10	C	4	C	6	16
6	8	C	14	6	C	12	C	8	14	6	C	12
7	4	C	16	14	C	8	16	C	4	14	8	C
8	8	C	6	16	2	C	8	C	16	2	16	C
9	6	2	C	C	10	14	C	14	10	6	C	2
10	C	14	4	2	12	C	4	C	14	2	12	C
11	4	C	8	C	14	6	C	8	4	14	C	6
12	C	10	2	16	C	12	C	10	2	C	12	16
13	10	8	C	12	C	4	10	8	C	C	4	12
14	10	C	6	16	14	C	16	14	C	C	10	6

[a]AM - Between 8:30 and 11:15 A.M.

[b]PM - Between 1:00 and 4:30 P.M.

[c]Controls (C) were randomly chosen from one of the age groups run in the same time period. All controls were treated with pure acetone. Tests were treated with 0.05 mg dimethyl phthalate dissolved in acetone.

Table 2

REPELLENCY INDICES WITH STATISTICAL CONFIDENCE LIMITS FOR AGE AND TIME OF DAY EFFECTS IN CONTROL AND REPELLENT-TREATED CASES

	Controls		Repellent Treated	
Age	Mean	95% C.L.	Mean	95% C.L.
2	73.4[bc]	55.7-91.2	2.0[a]	0-10.4
4	53.4[ab]	35.7-71.2	9.2[ab]	0.8-17.6
6	30.3[a]	12.6-48.0	3.8[a]	0-12.2
8	98.3[cd]	80.6-116.0	11.2[ab]	2.8-19.6
10	83.8[cd]	66.1-101.5	17.5[b]	9.1-25.9
12	99.0[d]	81.3-116.7	11.9[ab]	3.5-20.3
14	102.4[d]	84.7-120.1	16.9[b]	8.5-25.3
16	85.2[cd]	67.5-102.9	8.3[ab]	0-16.7
Time				
1st AM	61.9[a]	49.4-74.4	2.7[d]	0-8.6
2nd AM	71.2[ab]	58.7-83.7	11.1[b]	5.2-17.0
1st PM	81.8[bc]	69.3-94.3	15.4[b]	9.5-21.3
2nd PM	97.8[c]	85.3-110.3	11.2[b]	5.3-17.2

Superscripts a, b, c, and d represent statistical groups. Overlapping groups are denoted by more than one superscript in the mean values.

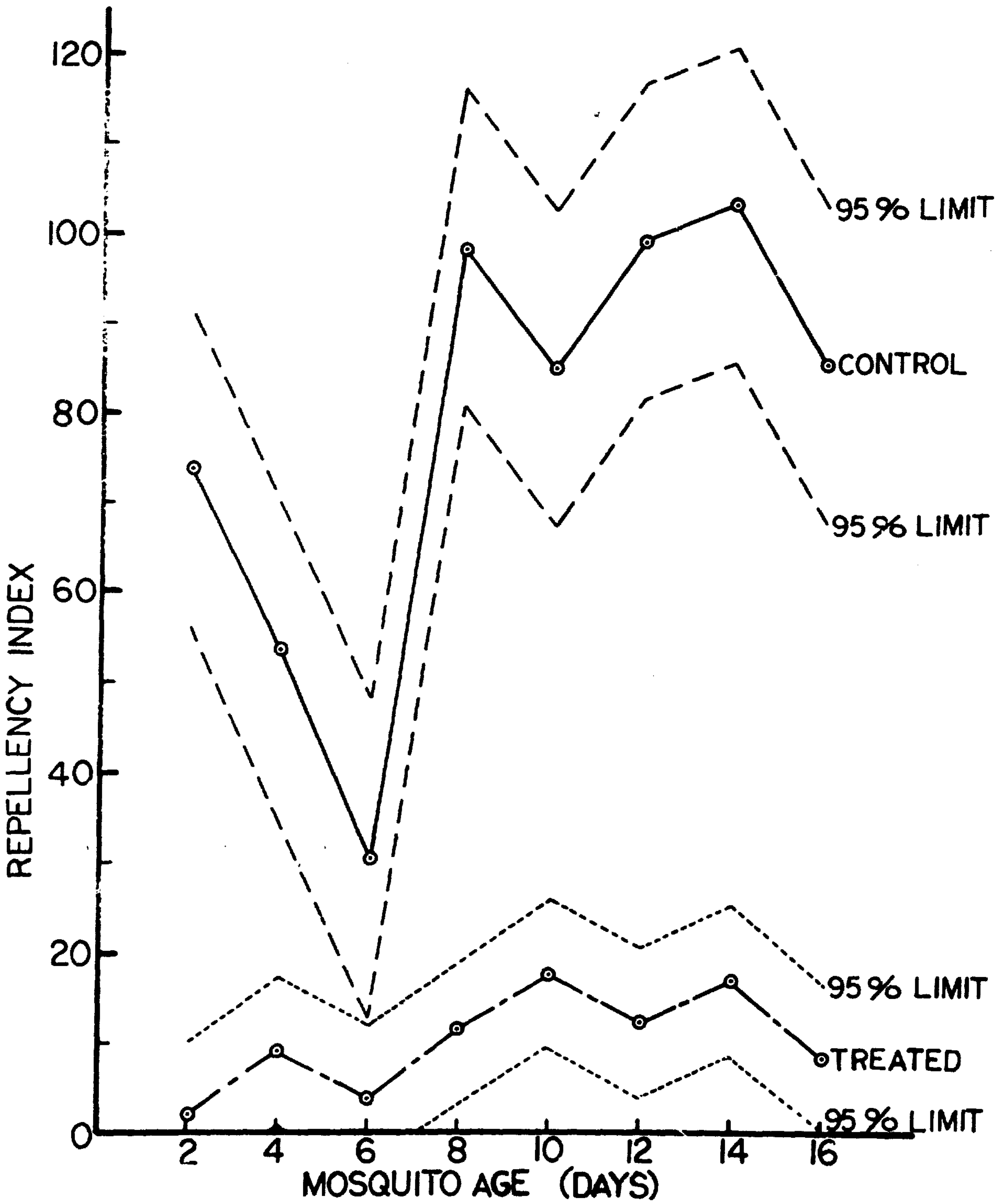


Figure 10

EFFECT OF MOSQUITO AGE ON THE REPELLENCY INDEX
OF CONTROL AND LOW-LEVEL REPELLENT-TREATED CASES

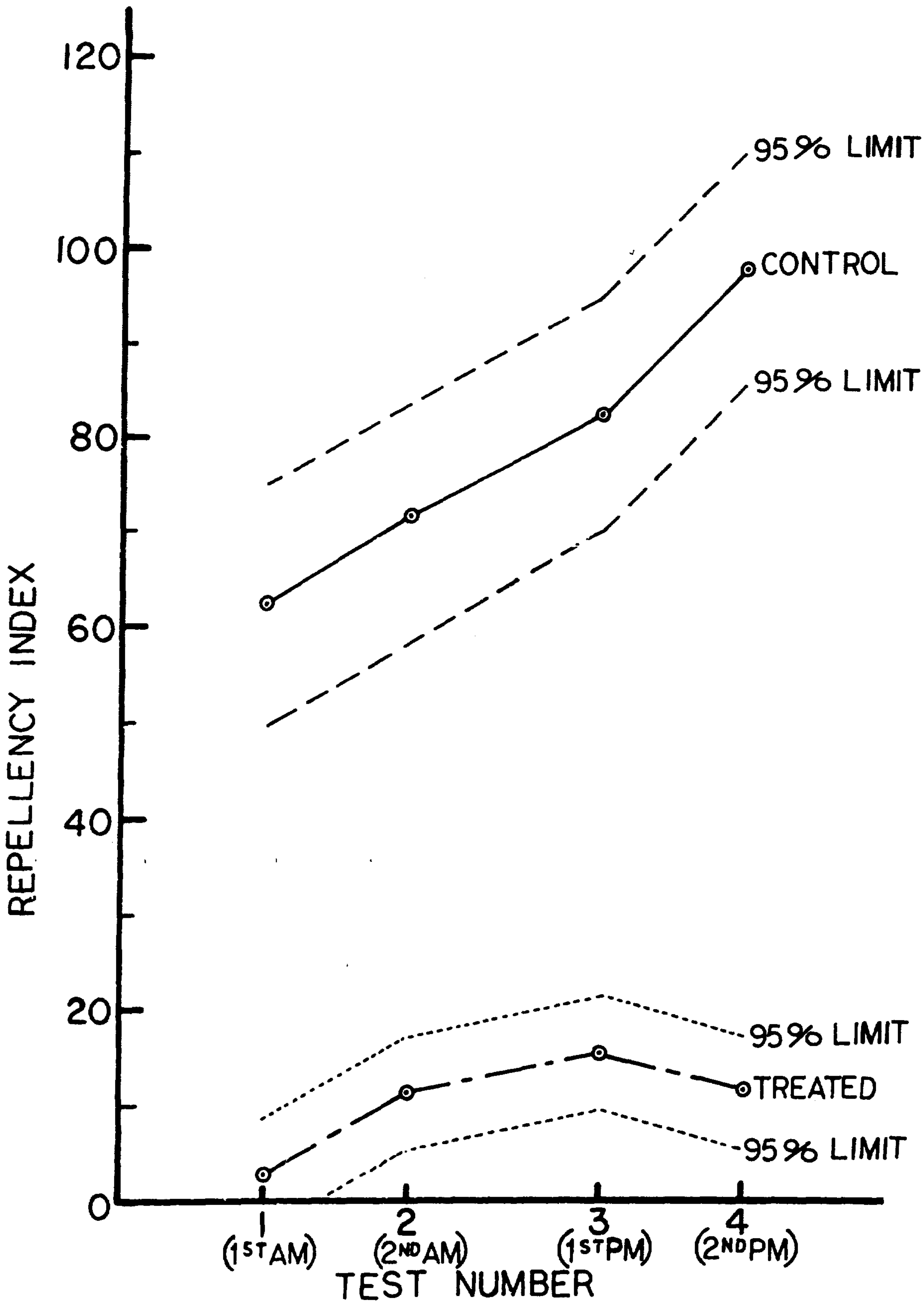


Figure 11

EFFECT OF TIME OF DAY ON THE REPELLENCY INDEX
OF CONTROL AND LOW-LEVEL REPELLENT-TREATED CASES

The confidence limits and the tests of significance were based upon the analysis of variance. Table 3 shows the analysis of variance of the repellency index for the controls. The day effect and age effect are significant at the 0.001 level, which means that this result could be the result of random variation with probability less than one-tenth of one percent, (i.e. it is probably not random). The effect for time of day of the experiment is also significant (0.005).

Common variation is not specifically associated with individual variables, but is accounted for by more than one variable. When it is negative the common variation requires that the variables are analyzed jointly to account for part of the sums of squares. Common variation can result from a randomized experimental design where some of the variables may not be completely balanced. When more variables than one are required to account for a portion of the sums of squares in an analysis of variance, then negative values of the common sums of squares can occur. These can be caused by random effects and can be safely ignored when they do not change the significance of variables in the analysis.

Table 4 shows the analysis of variance of the repellency index for the repellent treated test cases. The day effect and the time effect were significant at the 0.05 level. All other variables were not significant (n.s.).

No difference between meters was significant for either controls or test cases. The effect of accidental variations in the number of mosquitoes was tested and was not significant.

Although the repellency index, derived from disciminant function analysis, is the sum of the percent of actual biting time as electronically indicated (P) plus the percent mosquitoes engorged (E), it was also of interest to analyze these variables separately for the control cases. Comparison of Tables 5 and 6 shows that engorgement (Table 5) has the same significant variables as percent biting time (Table 6) but at lower levels of significance. Tables 5 and 6 show that the variables day, age, and time are significant for both P and for E.

Table 3

ANALYSIS OF VARIANCE OF REPELLENCY INDEX FOR CONTROLS

Source	d.f.	Sums of Squares	Mean Square	F	P
Day	13	29,649.1	2,280.7	4.340	$<$ .001
Age	7	19,728.7	2,818.4	5.360	$<$ .001
Meter	2	1,063.0	531.5	1.010	n.s.
Time	3	9,291.3	3,097.1	5.885	$<$ .005
No. of Mosquitoes	1	1.4	1.4	0.003	n.s.
Error	29	15,253.5	526.0		
Common	-	1,610.3			
Total	55	76,597.3			

Table 4

ANALYSIS OF VARIANCE OF REPELLENCY INDEX FOR REPELLENT-TREATED CASES

Source	d.f.	Sums of Squares	Mean Square	F	P
Day	13	5,670.4	436.2	1.845	$<$ .05
Age	7	2,505.3	357.9	1.509	n.s.
Meter	2	544.8	272.4	1.152	n.s.
Time	3	2,323.3	741.1	3.139	$<$ .05
No. of Mosquitoes	1	252.2	252.2	1.066	n.s.
Error	85	20,099.6	236.5		
Common	-	-222.7			
Total	111	31,172.9			

Table 5

ANALYSIS OF VARIANCE FOR ENGORGEMENT (E) OF CONTROLS

Source	d.f.	Sums of Squares	Mean Square	F	P
Day	13	3,967.6	305.20	1.812	$<.10$
Age	7	3,378.5	482.65	2.890	$<.025$
Meter	2	204.4	102.18	0.612	n.s.
Time	3	1,499.4	499.79	2.990	$<.05$
No. of Mosquitoes	1	54.4	54.36	0.325	n.s.
Error	29	4,842.0	166.96		
Total	55	13,946.2			

Table 6

ANALYSIS OF VARIANCE FOR PERCENT BITING TIME (P) OF CONTROLS

Source	d.f.	Sums of Squares	Mean Square	F	P
Day	13	11,948.9	921.91	3.030	$<.01$
Age	7	10,477.1	1,496.93	4.923	$<.005$
Meter	2	1,303.7	651.85	2.142	n.s.
Time	3	3,555.4	1,185.13	3.898	$<.025$
No. of Mosquitoes	1	38.7	38.70	0.127	n.s.
Engorgement (E)	1	155.2	155.20	0.510	n.s.
Error	28	8,522.2	304.36		
Total	55	36,037.2			

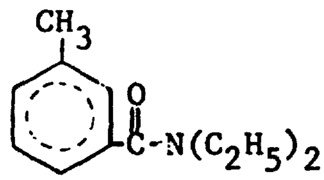
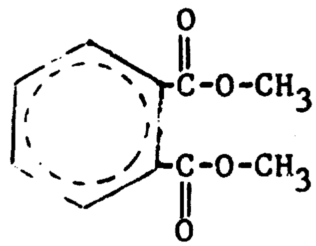
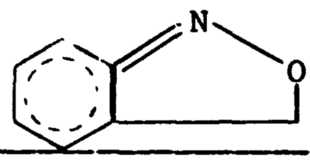
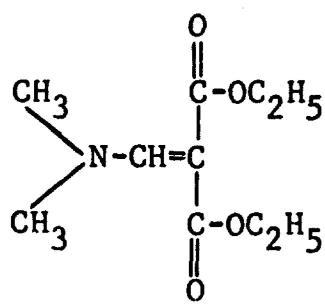
Table 6 also shows that after all these other variables are taken into account, the variable percent engorgement (E) is tested. The results show that the variables P and E are not significantly correlated except through the effect of the other variables. Thus, these two variables prove to give independent information which helps to provide more sensitive tests of the effect of repellent treatments.

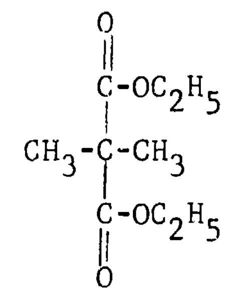
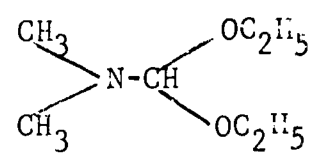
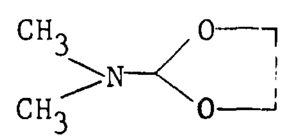
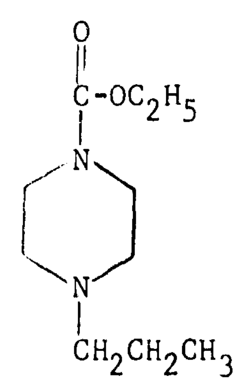
Gouck and Smith (ref. 15) found that avidity increased rapidly with age for the first 5 or 6 days, then remained fairly uniform. They also found that the morning avidity was much lower than during the previous afternoon, despite the additional age. Our results (Figure 10) show that there is a continual decrease in avidity up to about 6 days of age, and then a rapid increase after day 6. The same pattern appears with both control and repellent treated cases. Our results, however, agree with those of Gouck and Smith in our respective findings of variations of avidity with time of day. There is a decrease in avidity in the first morning test which is not significantly different from the second morning test (Table 2), but is highly significantly different from the afternoon tests. The second morning test is not significantly different from the first afternoon test. There appears to be a decrease in avidity in the second afternoon test in the repellent treated cases, and an increase in avidity during the same test period in controls (Figure 11). However, Table 2 shows that there is actually no significant differences for either the controls or the repellent-treated cases in these two time periods, since they fall into the same statistical groups.

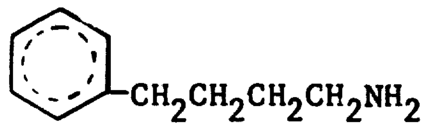
In conclusion, the results of these tests appear to substantiate the validity of all the assumptions implicit in this test method. The biting rate of mosquitoes on untreated controls parallels that of repellent-treated cases; there are no significant differences between the meters; the contribution of the parameter P is independent of that of E, and therefore provides unique information which contributes to the sensitivity of the overall repellent testing method.

IV. REPELLENCY ASSAY OF SELECTED COMPOUNDS

During this year a number of commercially available compounds were selected to be tested for repellency by the electronic recording method, together with the known repellents DEET and DMP for comparison. The compound names, computer listing, and structural formulas are as follows:

COMPOUND NAME	COMPOUND LISTING	STRUCTURE
m-Diethyltoluamide	DEET	
Dimethyl phthalate	DMP	
Anthranil	A14451-7	
Diethyl (Dimethylaminomethylene)-malonate	A14322-7	

COMPOUND NAME	COMPUTER LISTING	STRUCTURE
Diethyl Dimethylmalonate	A14390-1	
N,N-Dimethylformamide diethyl acetal	A14277-8	
N,N-Dimethylformamide ethylene acetal	A14275-1	
Ethyl-4-n-propyl-1-piperazine-carboxylate	A14154-2	
2-Methoxyethylamine	A14369-3	CH_3-O-$CH_2CH_2NH_2$

COMPOUND NAME	COMPUTER LISTING	STRUCTURE
3-Penten-2-one	A14501-7	$CH_3-CH=CH-\overset{\overset{\large O}{\|}}{C}-CH_3$
4-Phenylbutylamine	A14539-4	
2-Acetamido-3-butanone	A280-7	$CH_3-C(=O)-NH-CH(CH_3)-C(=O)-CH_3$

The computer outputs of the results of the repellency assays of these compounds are shown in the Appendix. It should be recalled that the number labeled "upper bound" gives the confidence limit for repellency of the compounds. Numbers below 100 for the upper bound are significantly repellent at the 95% level of confidence, numbers above 100 are not significantly repellent at this level of confidence. Relative merit is indicated by the magnitude of the upper bound. A compound with a low number indicates that it has better proof of repellency than a compound with a high number, ahtough both may be significantly repellent at the 95% level if both upper bounds are less than 100 (ref. 16).

It can be seen that both DEET and DMP are significantly repellent at an application level of 0.01 mg/sq in. of mouse skin. DEET may retain borderline repellency at a concentration of 0.001 mg/sq in., but DMP is not significantly repellent at this level.

It was previously reported that amino alcohols, amino aldehydes, and other compounds that appear to be related to the structure of GABA in terms of electronic distributions, i.e. having at least one nucleophilic center and one electrophilic center in the same molecule, usually show repellency at low treatment levels (ref. 2,3,4). Compounds that contain both nitrogen and oxygen atoms in certain relationships within the same molecule exhibit repellency. Also, compounds that contain a double bond between 2 C-atoms instead of amino nitrogen also provide a nucleophilic center, and show repellency when an electrophile is also present in the same molecule (ref. 3). The following compounds were tested to gain further insight into the chemical nature of a repellent.

Anthranil (computer listing A14451-7) showed no repellency at a concentration of 1.0 mg/sq in., and was not tested further. It is apparently detrimental to repellency to have both the nitrogen and oxygen atoms within a ring structure in which resonance and charge "smearing" can occur.

Diethyl (dimethylaminomethylene)-malonate (computer listing A14322-7) is significantly repellent at all 3 levels tested (1.0, 0.1, and 0.01 mg/sq in.). Here, two electron-releasing (nucleophilic) sites in the amino groups and the double bonded carbon atoms are complemented by two electron-withdrawing (electrophilic) groups in the diethyl malonate moieties, and the molecule exhibits significant repellency at the levels tested.

Diethyl dimethylmalonate (computer listing A14390-1) is not significantly repellent at either of the test levels observed (1.0 and 0.1 mg/sq in.). In this molecule, the electron-withdrawing (electrophilic) ester groups are not complemented by any electron-releasing groups, and the molecule is not repellent.

N,N-dimethylformamide diethyl acetal (computer listing A14277-8) shows significant repellency at both 1.0 and 0.1 mg/sq in. We have previously shown that amino acetals are fairly good repellents (ref. 2,3).

N,N-dimethylformamide ethylene acetal (computer listing A14275-1) shows no repellency at either the 1.0 mg/sq in. or the 0.1 mg/sq in. level. Apparently, when the oxygen atoms are in a saturated ring structure, it is very detrimental to the repellent properties of the molecule.

We next tested the repellency of ethyl 4-n-propyl-1-piperazine-carboxylate (computer listing A14154-2). In this molecule, 2 nitrogen atoms are in a 6-membered saturated ring, and 2 oxygen atoms are present in an ester linkage. We previously discussed the fact that when oxygen atoms were together in a molecule in an ester or an acetal linkage, repellency was retained. These findings were interpretated in terms of their similarities and dissimilarities to the structure of GABA, which contains one strongly electrophilic group (the carboxyl group) and one strongly nucleophilic group (the amino group). The repellency of a substance could be shown if the nitrogen atom was surrounded by carbon atoms, but not the oxygen atom (ref. 2,3). In the compound ethyl 4-n-propyl-1-piperazine-carboxylate, nitrogen atoms are surrounded by carbon atoms in a ring structure, and two oxygen atoms are present in an ester linkage. This compound is significantly repellent at the 1.0 and 0.1 mg/sq in. levels. Thus, although this compound is totally different structurally from many other repellent compounds we have tested, comparison of the test results of this compound with that of N,N-dimethyl formamide ethylene acetal (A14275-1) seems to bear out the basic principle that modification of the electrophilic properties of oxygen by insertion into a carbon chain will significantly reduce repellency, while the nucleophilic properties of nitrogen are not greatly modified by other electron-releasing groups, since the amino group is itself electron-releasing.

An interesting possible departure from this rule is seen in the compound 2-methoxyethylamine (computer listing A14369-3). Here, the oxygen is surrounded by carbon atoms, but it is in the form of a methoxy ether-linkage. This is the first amino ether we have tested. This compound proved to be significantly repellent at all levels tested (1.0, 0.1, 0.01 mg/sq in.). It will be of interest to further investigate the repellent properties of compounds with this type of structure.

We previously reported that 3-butene-2-ol was significantly repellent at an application level of .01 mg/sq in. (ref. 3). In this series of tests, we tested the repellency of 3-penten-2-one (computer listing A14501-7). This compound showed no repellency at an application level of 1.0 mg/sq in. and was not tested further. In this compound there is the possibility of resonance between the unsaturated double bonded carbon atoms and the keto-group. Such resonance would tend to "smear" out the charge difference between these two groups, and thus vitiate the charge separation between the nucleophile and electrophile (i.e., between the double-bonded -C=C- group and the keto-group respectively). Under these conditions, repellency is apparently completely lost. Structurally the compounds 3-butene-2-ol and 3-penten-2-one are not very dissimilar. This finding again tends to substantiate the hypothesis that the electronic distribution in a molecule rather than its actual atomic constituents is the main factor that determines the repellency of a compound.

4-Phenylbutylamine (computer listing A14539-4) was next tested for repellency. This compound was found to be significantly repellent at concentrations of 1.0 and 0.1 mg/sq in. but not at lower levels. Though the existence of the nucleophile (the amino group) is evident, this compound cannot easily be shown to have complementary nucleophilic and electrophilic centers. This 4-carbon compound, however, may have a steric resemblence to the structure of GABA. The evident and unexpected repellency of this compound demonstrates that there may exist more than one mechanism by which a substance may cause avoidance reactions in a mosquito.

The last compound, 2-acetamido-3-butanone (computer listing A280-7) is of special interest. In view of the physiological action of N-acetyl-GABA (previously shown in the cockroach preparation), it was of interest to test a volatile analogue of NAG for repellency, namely N-acetyl-4-butanol (NAB). Upon consulting chemical catalogues and chemical literature, we found that this compound is not commercially available, and indeed, has never been reported in the literature. We therefore chose 2-acetamido-3-butanone to test for repellency, since it seemed to come closest to the desired structure and is commercially available. Although 2-acetamido-3-butanone is a ketone, and we previously found ketones not to be very repellent, this compound retained significant repellency to a concentration of 0.1 mg/sq in. Though it was not repellent below this level, these results are very encouraging. If possible, we will attempt in future work to synthesize NAB to test for repellency. If it is repellent, the mechanism by which it exerts repellency will seem clear. By analogy to the highly stimulatory effects that we found NAG to exert in the cockroach central nervous system, NAB would increasingly stimulate the neural receptor structures of the mosquito as a prospective host is approached, and thus unbalance and confuse the delicate mechanism governing the host-finding activities of the mosquito.

V. SUMMARY

A. Electrophysiological Investigations

During this year detailed systematic studies were conducted to test the hypothesis that gamma-aminobutyric acid (GABA), a neuroinhibitory substance that is found in mosquito extracts, becomes neuroexcitatory when it reacts with carbon dioxide to form a carbamino compound. It was shown that treatment of the isolated cockroach central nervous system with GABA reduces its rate of spontaneous bioelectric discharge. When the preparation is treated with CO_2 alone, or with a GABA solution that had 10% CO_2 bubbled into it, the spontaneous discharge rate increases.

In order to distinguish between the stimulatory action of CO_2 alone and carbamino-GABA, the effect of gamma-hydroxybutyric acid (GHB) was observed in the presence and absence of CO_2. GHB cannot form a carbamino compound since it does not contain an amino group. It was found that in the presence of GHB the sensitivity of the preparation to CO_2 is completely abolished. This result implies that carbamino-GABA formation is the only mechanism by which CO_2 acts as a neurostimulator in this preparation. A stable chemical analogue of carbamino-GABA, N-acetyl GABA (NAG) was applied to the preparation, and found to be neurostimulatory. Competition studies between NAG and GABA showed that both these substances appear to compete for the same site on the synaptic membrane. This observation provides additional evidence that carbamino-GABA formation is the mechanism by which CO_2 stimulates the insect central nervous system, and does not act through a separate receptor site for CO_2. These results were related to the possible mechanism by which CO_2 exerts central and local control of ventilation in insects, and triggers the host-seeking activities of mosquitoes.

B. Studies of Repellency Assay by the Electronic Method

A statistically designed series of tests were performed to test the assumptions that (1) variables affecting the biting rate of mosquitoes on untreated control mice will also affect the repellent-treated cases in a parallel way; (2) there are no significant differences in the data output among the three meters used in the electronic recording method; (3) the electronically recorded data makes a significant and independent contribution to the total assay of repellency. These three assumptions were statistically proven to be correct, and the validity of the electronic repellency assay method was thus further documented.

C. Tests of Repellents

A number of compounds were tested for repellency that were selected to further test the rationale that if a substance is volatile, and has an electronic structure similar to that of GABA, i.e., contains at least one nucleophilic and one electrophilic center, it will be repellent. Though an exception was noted, it was shown that in general substances that had this electronic structure were repellent, while substances that did not have this structure were not repellent. This further substantiated previous findings. The possibility was discussed that volatile chemical analogues of another physiologically active substance discovered in the course of this work, N-acetyl GABA, may also be considerably repellent to mosquitoes.

VI. FUTURE INVESTIGATIONS

The approaches described above and detailed in past reports will as far as possible be continued in future work. We anticipate that the future work program will encompass the following areas of investigation.

A. Further Investigations of the GABA Hypothesis

Work will continue in the general avenues of research described in this report. We will further investigate the identity or non-identity of the sites of action of GABA, GABA-CO_2, GHB, and glutamate in the insect central nervous system. The encouraging positive results thus far obtained with the cockroach preparation will be further explored and substantiated.

B. Search for New Repellents

The search for new repellents based upon specific requirements in terms of molecular and electronic configurations of candidate compounds will continue. Commercially available materials will be obtained whenever possible to continue to test these approaches. If possible, we will attempt to synthesize the alcohol analogue of N-acetyl GABA. The latter substance shows potent physiological activity in the insect central nervous system. This alcohol analogue, 4-acetamidobutanol, may exhibit substantial repellency.

C. Maintenance of Mosquito and Cockroach Colonies

Our colonies of *Aedes aegypti* (L) mosquitoes and *Periplaneta americana* (L) cockroaches will continue to be propagated and maintained for purposes of our research throughout the course of this work.

VII. CONCLUSIONS

Our investigations to date have included 3 main areas of research:

1. Testing a hypothesis that could explain the basic physiological mechanisms that drive mosquitoes to warm-blooded hosts.

2. Developing accurate statistically-based methods to evaluate mosquito repellency of test compounds. These methods employ a device that electronically records, and thus unequivocally documents the mosquito bite.

3. Testing the mosquito repellency of certain chemical structures that are related to the structure of GABA and exploring the nature of a repellent and the mechanism of repellency. This work is directed toward obtaining a rationale to guide the discovery of more potent and longer-lasting repellents than those currently available.

We plan to continue using the unique theoretical and methodological approaches developed during the course of this work. The attempt to elucidate the basic physiological mechanisms governing the interactions of mosquitoes with their warm-blooded hosts, and the logical approaches to the development of new insect repellents engendered during this work are bearing fruitful results. Continuation of this effort should substantially contribute to ultimate success in the important endeavor to achieve superior repellents for systemic or topical use.

LITERATURE CITED

1. Kashin, P., "Development of an Orally Effective Insect Repellent," Annual Progress Report No. IITRI-L6021-4, Contract No. DA-49-193-MD-2281 conducted by IIT Research Institute, Chicago, Illinois, October 31, 1965.

2. Kashin, P., "Development of an Orally Effective Insect Repellent," Annual Progress Report No. IITRI-L6021-8, Contract No. DA-49-193-MD-2281 conducted by IIT Research Institute, Chicago, Illinois, November 11, 1966.

3. Kashin, P., "Development of an Orally Effective Insect Repellent," Annual Progress Report No. IITRI-L6021-12, Contract No. DA-49-193-MD-2281 conducted by IIT Research Institute, Chicago, Illinois, November 1967.

4. Kashin, P., "Development of an Orally Effective Insect Repellent," Annual Progress Report No. IITRI-L6021-16, Contract No. DA-49-193-MD-2281 conducted by IIT Research Institute, Chicago, Illinois, November 1968.

5. Kashin, P., Ann. Entomol. Soc. of Amer. 62, 695 (1969).

6. Smit, W. A., Becht, G., and Beenakkers, A. M. Th., J. Insect. Physiol. 13, 1857 (1967).

7. Edwards, C. and Kuffler, S. W., J. Neurochem. 4, 19 (1959).

8. Margolis, R. K., Biochem. Pharmacol. 18, 1243 (1969).

9. Kashin, P., Quarterly Progress Report, IITRI-L6021-17, January 31, 1969.

10. Jensen, A. and Faurhalt, C., Acta Chem. Scand. 6, 385, (1952).

11. Herford, G. M., J. Exptl. Biol. 15, 327 (1938).

12. Edwards, G. A., Respiratory Mechanisms, in Roeder, K. D., Insect Physiology, John Wiley-Son, Publ., New York (1953).

13. Guthrie, D. M. and Tindall, A. R., Respiration (Chapt. 16), in, The Biology of the Cockroach, St. Martin's Press, Publ. New York (1968).

14. Kerkut, G. A., Leake, L. D., Cowan, S., Shapiro, A., and Walker, R., J. Comp. Biochem. Physiol. 15, 485 (1965).

15. Gouck, H. K. and Smith, C. N., The Florida Entomologist 45, 93 (1963).

16. Kashin, P. and Kardatzke, M. L., J. Econ. Entomol. 62, 10 (1969).

17. Kashin, P. and Arneson, B. E., J. Econ. Entomol. 62, 200 (1969).

APPENDIX

REPELLENCY ASSAY OF SELECTED COMPOUNDS

CONTROL VALUES

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS	Day of Test	Test Number
	-0.00000	17.31	49.97	67.27		1	1
	-0.00000	41.18	80.53	121.71		1	2
	-0.00000	42.00	99.13	141.13		1	3
	-0.00000	18.52	95.17	113.69		1	4
	-0.00000	19.61	54.21	73.81		2	5
	-0.00000	33.33	86.07	119.40		2	6
	-0.00000	49.02	89.97	138.99		3	7
	-0.00000	28.00	88.00	116.00		3	8
	-0.00000	61.70	94.90	156.60		3	9
	-0.00000	44.23	70.60	114.83		4	10
	-0.00000	29.41	90.60	120.01		4	11
	-0.00000	68.63	87.17	155.79		4	12
	-0.00000	35.56	68.43	103.99		5	13
	-0.00000	67.35	92.77	160.11		5	14
	-0.00000	23.53	37.70	61.23		5	15
	-0.00000	30.91	90.13	121.04		6	16
	-0.00000	51.79	95.40	147.19		6	17
	-0.00000	20.75	83.47	104.22		6	18
	-0.00000	33.33	80.20	113.53		7	19
	-0.00000	21.74	82.37	104.11		7	20
	-0.00000	24.53	70.67	95.19		8	21
	-0.00000	41.18	80.83	122.01		8	22
	-0.00000	70.91	85.97	156.88		8	23
	-0.00000	20.75	83.07	103.82		9	24
	-0.00000	20.75	83.17	103.92		9	25
	-0.00000	39.62	36.67	76.29		10	26
	-0.00000	44.44	49.57	94.01		10	27
	-0.00000	45.10	94.77	139.86		10	28
	-0.00000	21.82	50.13	71.95		11	29
	-0.00000	27.45	73.30	100.75		11	30
	-0.00000	53.70	98.47	152.17		11	31
	-0.00000	33.33	55.40	88.73		12	32
	-0.00000	31.37	64.10	95.47		12	33
	-0.00000	48.21	95.97	144.18		12	34
	-0.00000	60.00	72.20	132.20		13	35
	-0.00000	28.21	71.10	99.31		13	36
	-0.00000	28.57	44.07	72.64		14	37
	-0.00000	75.51	87.07	162.58		14	38
	-0.00000	86.36	93.57	179.93		14	39
	-0.00000	38.78	51.90	90.68		15	40
	-0.00000	17.78	48.17	65.94		15	41
	-0.00000	30.00	61.10	91.10		16	42
	-0.00000	54.35	65.33	119.68		16	43
	-0.00000	60.38	92.83	153.21		16	44

CONTROL VALUES (cont.)

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ. INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS		Day of Test	Test Number
	-0.00000	37.04	48.27	85.30			17	45
	-0.00000	64.15	72.07	136.22			17	46
	-0.00000	44.00	92.50	136.50			18	47
	-0.00000	36.00	55.57	91.57			18	48
	-0.00000	21.74	26.63	48.37			19	49
	-0.00000	31.11	34.03	65.14			19	50
	-0.00000	43.45	69.73	115.19			20	51
	-0.00000	47.83	83.93	131.76			20	52
	-0.00000	46.94	76.20	123.14			21	53
	-0.00000	62.50	75.47	137.97			21	54
	-0.00000	65.31	86.87	152.17			22	55
	-0.00000	29.17	38.57	67.73			22	56
	-0.00000	34.69	51.50	86.19			23	57
	-0.00000	43.86	74.03	117.89			24	58
	-0.00000	28.00	48.13	76.13			24	59
	-0.00000	47.92	72.90	120.82			25	60
	-0.00000	23.40	34.80	58.20			25	61
	-0.00000	55.56	68.87	124.42			26	62
	-0.00000	31.11	34.03	65.14			27	63
	-0.00000	21.74	26.63	48.37			27	64
	-0.00000	47.83	83.93	131.76			28	65
	-0.00000	47.83	83.93	131.76			28	66
	-0.00000	46.30	48.33	94.63			29	67
	-0.00000	69.23	70.40	139.63			29	68
	-0.00000	16.67	55.70	72.37			30	69
	-0.00000	17.31	76.83	94.14			31	70
	-0.00000	26.00	63.23	89.23			32	71
CONTROL	-0.00000	39.85	70.13	109.99	100.0	-0.0000=CONTRAST		
		16.53	19.96	31.26	(110.0)UPPER BOUND	5.4127=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS		Day of Test	Test Number
DEET								
	0.10000	0.00	0.03	0.03			18	1
	0.10000	0.00	0.07	0.07			19	2
	0.10000	0.00	2.53	2.53			20	3
DEET	0.10000	0.00	0.88	0.88	0.9	97.2107=CONTRAST		
		-0.00	1.43	1.43	(47.9)UPPER BOUND	22.8042=STANDARD ERROR		
DEET								
	0.01000	5.68	12.57	18.45			19	1
	0.01000	2.08	3.83	5.92			19	2
	0.01000	25.00	52.77	77.77			20	3
DEET	0.01000	10.99	23.06	34.04	34.7	64.0444=CONTRAST		
		12.28	26.10	38.38	(81.7)UPPER BOUND	22.8042=STANDARD ERROR		
DEET								
	0.00100	41.18	60.13	101.31			21	1
	0.00100	16.00	44.97	60.97			21	2
	0.00100	18.75	50.07	68.82			22	3
	0.00100	13.46	43.33	56.79			22	4
DEET	0.00100	22.35	49.62	71.97	59.9	48.2809=CONTRAST		
		12.74	7.57	20.18	(58.2)UPPER BOUND	22.8042=STANDARD ERROR		
DMP								
	0.05000	5.88	3.73	9.62			30	1
	0.05000	7.84	21.63	29.48			31	2
	0.05000	2.00	9.47	11.47			32	3
DMP	0.05000	5.24	11.61	16.85	19.8	66.3941=CONTRAST		
		2.97	9.14	10.97	(82.3)UPPER BOUND	26.3321=STANDARD ERROR		

REPELLENCY OF CCMPOUNCS CCNTRASTED WITH CCNTRCL VALUES

CCMPCLNC NAME	CONCENTRATICN CN POLSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CCNTROLS		Day of Test	Test Number
DMF								
	C.C1CCC	20.41	59.57	79.97			9	1
	C.C1CCC	3.77	12.40	16.17			10	2
	0.C1CCC	2.17	15.43	17.61			12	3
DMP	C.C10CC	8.79	29.13	37.92	34.4	65.3385=CONTRAST		
		10.10	26.40	36.43	(76.3)UPPER BOUND	21.9096=STANDARD ERROR		
DMF								
	C.CC5CC	29.09	63.27	92.36			10	1
	0.CC5CC	16.67	45.27	61.93			11	2
	C.CC5CC	8.82	26.37	35.19			11	3
	C.CO5CC	7.41	65.27	72.67			12	4
DMP	0.CC5CC	15.50	50.04	65.54	62.9	39.8294=CONTRAST		
		9.94	18.17	23.83	(99.9)UPPER BOUND	19.6268=STANDARD ERROR		
DMP								
	C.C01CC	44.44	62.10	106.54			9	1
	0.CO1CC	33.33	67.07	100.40			10	2
	C.CO1CC	9.62	29.20	38.82			12	3
DMP	C.CO1CC	29.13	52.79	81.92	76.7	24.6666=CONTRAST		
		17.79	20.58	37.46	(118.6)UPPER BOUND	21.9096=STANDARD ERROR		
A14451-7								
	1.CCCCC	23.C8	63.33	86.41			6	1
	1.CCCCC	54.72	88.77	143.48			8	2
	1.CCCCC	46.51	53.C7	99.58			13	3
A14451-7	1.C00CC	41.44	68.39	109.82	90.5	11.5361=CONTRAST		
		16.42	18.38	29.88	(126.9)UPPER BOUND	21.9096=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON POLSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS		Day of Test	Test Number
A14322-7								
	1.00000	2.53	14.63	17.26			13	1
	1.00000	6.25	33.80	40.05			14	2
	1.00000	13.73	10.30	24.03			14	3
	1.00000	13.89	16.53	30.42			15	4
	1.00000	0.00	0.03	0.03			16	5
A14322-7	1.00000	7.30	15.06	22.36	18.2	96.2952=CONTRAST		
		6.34	12.27	15.04	(48.8)UPPER BOUND	17.7981=STANDARD ERROR		
A14322-7								
	0.10000	2.22	42.10	44.32			13	1
	0.10000	11.54	23.40	34.94			14	2
A14322-7	0.10000	6.88	32.75	39.63	30.8	66.3784=CONTRAST		
		6.59	13.22	6.64	(73.7)UPPER BOUND	27.0955=STANDARD ERROR		
A14322-7								
	0.01000	13.73	46.87	60.59			16	1
	0.01000	23.06	72.87	95.94			17	2
	0.01000	5.88	10.50	16.38			18	3
	0.01000	10.20	28.67	38.87			20	4
A14322-7	0.01000	13.22	39.72	52.95	45.3	64.3398=CONTRAST		
		7.31	26.62	33.87	(78.7)UPPER BOUND	19.4475=STANDARD ERROR		
A14390-1								
	1.00000	33.93	78.77	112.70			6	1
	1.00000	32.00	80.53	112.53			8	2
	1.00000	12.50	60.23	72.73			11	3
A14390-1	1.00000	26.14	73.18	99.32	83.4	19.7241=CONTRAST		
		11.85	11.24	23.03	(120.0)UPPER BOUND	21.5001=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ. INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
A1439C-1								
	0.10000	38.00	79.57	117.57			1	1
	0.10000	16.28	12.33	28.61			4	2
	0.10000	23.53	67.07	90.60			6	3
A14390-1	0.10000	25.34	52.99	78.93	65.6	41.7706=CONTRAST		
		11.06	35.76	45.61	(101.0)UPPER BOUND	21.2651=STANDARD ERROR		
A14277-6								
	1.00000	7.69	20.97	28.66			5	1
	1.00000	41.51	43.80	85.[illegible]1			7	2
	1.00000	18.75	41.43	60.18			8	3
	1.00000	14.55	19.43	33.98			17	4
A14277-8	1.00000	20.62	31.41	52.03	45.5	61.7939=CONTRAST		
		14.65	12.99	26.12	(79.7)UPPER BOUND	19.1594=STANDARD ERROR		
A14277-6								
	0.10000	0.00	0.20	0.20			1	1
	0.10000	20.83	67.43	88.27			1	2
	0.10000	26.32	53.93	80.25			2	3
	0.10000	30.61	77.03	107.65			3	4
	0.10000	44.68	39.00	83.68			4	5
A14277-6	0.10000	24.49	47.52	72.01	62.0	44.8353=CONTRAST		
		16.29	30.09	41.52	(91.6)UPPER BOUND	17.2384=STANDARD ERROR		
A14275-1								
	1.00000	17.31	48.33	65.64			5	1
	1.00000	23.08	64.73	87.81			8	2
	1.00000	5.77	75.67	81.44			11	3
A14275-1	1.00000	15.38	62.91	78.30	68.8	35.5138=CONTRAST		
		8.81	13.76	11.41	(107.0)UPPER BOUND	21.5001=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON POLSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS		Day of Test	Test Number
A14275-1								
	0.10000	1.85	4.90	6.75			1	1
	0.10000	52.94	63.07	116.01			2	2
	0.10000	23.49	96.43	121.92			3	3
	0.10000	23.41	82.27	111.68			4	4
A14275-1	0.10000	27.42	61.67	89.09	72.9	32.2855=CONTRAST		
		20.92	40.24	55.05	(104.7)UPPER BOUND	18.7239=STANDARD ERROR		
A14154-2								
	1.00000	0.00	0.83	0.83			6	1
	1.00000	0.00	5.50	5.50			7	2
	1.00000	0.00	0.17	0.17			8	3
A14154-2	1.00000	0.00	2.17	2.17	1.7	117.5828=CONTRAST		
		-0.00	2.91	2.91	(38.8)UPPER BOUND	21.9096=STANDARD ERROR		
A14154-2								
	0.10000	19.61	69.17	88.77			1	1
	0.10000	15.22	63.70	78.92			3	2
	0.10000	7.14	43.63	50.78			4	3
A14154-2	0.10000	13.99	58.83	72.82	58.2	52.6204=CONTRAST		
		6.32	13.44	19.72	(92.4)UPPER BOUND	21.2651=STANDARD ERROR		
A14365-3								
	1.00000	5.26	5.67	10.93			8	1
	1.00000	15.69	89.17	104.85			12	2
	1.00000	7.69	29.57	37.26			12	3
	1.00000	5.26	3.57	8.83			16	4
A14365-3	1.00000	8.48	31.99	40.47	31.7	79.9207=CONTRAST		
		4.94	39.90	44.83	(65.6)UPPER BOUND	19.6268=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS		Day of Test	Test Number
A14365-3								
	0.10000	5.36	17.33	22.69			1	1
	0.10000	3.77	56.37	60.14			2	2
	0.10000	6.12	22.83	28.96			3	3
	0.10000	9.80	62.83	72.64			4	4
A14365-3	0.10000	6.26	39.84	46.11	38.0	73.9150=CONTRAST		
		2.55	23.08	24.11	(69.8)UPPER BOUND	18.7239=STANDARD ERROR		
A14365-3								
	0.01000	35.42	76.33	111.75			5	1
	0.01000	12.96	28.53	41.50			12	2
	0.01000	11.36	32.83	44.20			16	3
A14365-3	0.01000	19.91	45.90	65.81	58.2	47.2645=CONTRAST		
		13.45	26.44	39.80	(96.7)UPPER BOUND	21.5001=STANDARD ERROR		
A14501-7								
	1.00000	34.62	33.63	68.25			27	1
	1.00000	17.78	62.37	80.14			28	2
	1.00000	53.06	76.07	129.13			29	3
A14501-7	1.00000	35.15	57.36	92.51	90.8	9.3757=CONTRAST		
		17.65	21.66	32.27	(136.1)UPPER BOUND	22.8042=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON POLSE (MG/SQ.INCH)	MOSQUITOES ENGORGED PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WIEGHTED PERCENT OF CONTROLS		Day of Test	Test Number
A14539-4								
	1.00000	0.00	3.47	3.47			14	1
	1.00000	0.00	6.00	6.00			15	2
	1.00000	0.00	16.27	16.27			16	3
	1.00000	0.00	17.50	17.50			17	4
A14539-4	1.00000	0.00	10.81	10.81	9.5	102.4817=CONTRAST		
		-0.00	7.11	7.11	(43.7)UPPER BOUND	19.1594=STANDARD ERROR		
A14539-4								
	0.10000	0.00	13.43	13.43			1	1
	0.10000	5.66	4.27	9.93			2	2
	0.10000	19.61	35.90	55.51			3	3
	0.10000	0.00	5.07	5.07			4	4
	0.10000	17.65	57.67	75.31			17	5
A14539-4	0.10000	8.58	23.27	31.85	26.4	86.5754=CONTRAST		
		9.48	23.09	31.57	(55.5)UPPER BOUND	16.9191=STANDARD ERROR		
A14539-4								
	0.01000	75.47	79.13	154.61			14	1
	0.01000	32.08	79.20	111.28			15	2
	0.01000	17.02	58.23	75.25			16	3
	0.01000	23.08	39.33	62.41			17	4
A14539-4	0.01000	36.91	63.97	100.89	89.8	11.5222=CONTRAST		
		26.44	19.16	41.36	(124.1)UPPER BOUND	19.1594=STANDARD ERROR		

REPELLENCY OF COMPOUNDS CONTRASTED WITH CONTROL VALUES

COMPOUND NAME	CONCENTRATION ON MOUSE (MG/SQ.INCH)	MOSQUITOES ENGORGED (PCT)	TIME DISPLACED (PCT)	REPELLENCY INDEX	WEIGHTED PERCENT OF CONTROLS		Day of Test	Test Number
A280-7								
	1.00000	2.34	12.23	14.27			21	1
	1.00000	4.35	14.63	18.98			2[illegible]	2
	1.00000	1.96	21.43	23.39			23	3
	1.00000	0.00	0.03	0.03			24	4
A280-7	1.00000	2.09	12.08	14.17	12.6	9[illegible].6380=CONTRAST		
		1.78	9.93	10.13	(51.1)UPPER BOUND	20.3967=STANDARD ERROR		
A280-7								
	0.10000	4.26	13.37	17.62			21	1
	0.10000	3.77	9.33	13.11			22	2
	0.10000	3.57	20.53	24.10			23	3
	0.10000	1.89	6.97	8.85			24	4
A280-7	0.10000	3.37	12.55	15.92	14.3	91.8676=CONTRAST		
		1.03	5.94	6.53	(52.8)UPPER BOUND	20.3967=STANDARD ERROR		
A280-7								
	0.01000	23.08	68.37	91.44			24	1
	0.01000	18.00	48.67	66.67			25	2
	0.01000	23.08	68.00	91.08			26	3
A280-7	0.01000	21.38	61.68	83.06	80.9	19.4263=CONTRAST		
		2.93	11.27	14.20	(128.3)UPPER BOUND	23.8183=STANDARD ERROR		
A280-7								
	0.00100	10.91	32.53	43.44			24	1
	0.00100	54.35	77.73	132.08			25	2
	0.00100	62.50	78.43	140.93			26	3
A280-7	0.00100	42.59	62.90	105.49	100.5	-0.5030=CONTRAST		
		27.73	26.30	53.91	(147.8)UPPER BOUND	23.8183=STANDARD ERROR		

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13. ABSTRACT

Experimental observations were made to test the validity of the gamma-aminobutyric acid (GABA)-carbon dioxide (CO_2) hypothesis, designed to explain the neurochemical interactions that govern a mosquito's behavior in the presence of its host. It was postulated that the formation of carbamino-GABA underlies the activating effects of CO_2 on mosquitoes.

The isolated central nervous system of the cockroach, P. americana, was utilized to test the effects of carbamino-GABA and other compounds on the nervous system of an insect. GABA inhibited the spontaneous firing rate of this preparation in the absence of CO_2, but stimulated it in its presence. Gamma-hydroxybutyric acid, a substance which cannot form a carbamino compound, was inhibitory both in the absence and presence of CO_2. A chemical analogue of carbamino-GABA, N-acetyl GABA stimulated the preparation. It was shown that NAG and GABA probably compete for the same site on the synaptic membrane. Further studies are in progress.

The evaluation of selected compounds for mosquito repellency by the electronic recording method was continued. Compounds were selected on the basis of our past approach, i.e. that volatile chemical analogues of GABA would affect the mosquito's responses to its host.

A statistically designed series of tests were performed to test the validity of some basic assumptions that are involved in using the electronic method for the assay of repellents. The results of these tests largely substantiated our confidence in this method.

14 KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Mosquito						
Repellent						
Gamma-aminobutyric acid						
Carbon dioxide						
Neuroinhibitor						
Activation						
Electronic						
Bitometer-Timer						
Repellency index						
Computerized repellency assay						